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INTEGRINS IN TUMORIGENESIS AND CANCER CELL INVASION

by

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To my family

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Integrins in Tumorigenesis and Cancer Cell Invasion
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ABSTRACT

The integrin family of transmembrane receptors are important for cell-matrix adhesion and signal transmission to the interior of the cell. Integrins are essential for many physiological processes and defective integrin function can consequently result in a multitude of diseases, including cancer. Integrin traffic is needed for completion of cytokinesis and cell division failure has been proposed to be an early event in the formation of chromosomally aberrant and transformed cells. Impaired integrin traffic and changes in integrin expression are known to promote invasion of malignant cells. However, the direct roles of impaired integrin traffic in tumorigenesis and increased integrin expression in oncogene driven invasion have not been examined. In this study we have investigated both of these aspects.

We found that cells with reduced integrin endocytosis become binucleate and subsequently aneuploid. These aneuploid cells display characteristics of transformed cells; they are anchorage-independent, resistant to apoptosis and invasive *in vitro*. Importantly, subcutaneous injection of the aneuploid cells into athymic nude mice produced highly malignant tumors. Through gene expression profiling and analysis of integrin-triggered signaling pathways we have identified several molecules involved in the malignancy of these cells, including Src kinase and the transcription factor Twist2. Thus, even though chromosomal aberrations are associated with reduced cell fitness, we show that aneuploidy can facilitate tumor evolution and selection of transformed cells.

Invasion and metastasis are the primary reason for deaths caused by cancer and the molecular pathways responsible for invasion are therefore attractive targets in cancer therapy. In addition to integrins, another major family of adhesion receptors are the proteoglycans syndecans. Integrins and syndecans are known to signal in a synergistic manner in controlling cell adhesion on 2D matrixes. Here we explored the role of syndecans as α2β1 integrin co-receptors in 3D collagen. We show that in breast cancer cells harbouring mutant K-Ras, increased levels of integrins, their co-receptors syndecans and matrix cleaving proteases are necessary for the invasive phenotype of these cells.

Together, these findings increase our knowledge of the complicated changes that occur during tumorigenesis and the pathways that control the ability of cancer cells to invade and metastasize.

Keywords: cancer, aneuploidy, cell invasion, integrin, syndecan, Twist2, Src

Gunilla Högnäs
Integriinien merkitys syövän synnyssä ja syöpäsolujen invaasiossa
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TIIVISTELMÄ

Integriinit ovat solukalvon läpäiseviä tarttumisreseptoreja, joiden avulla solu muodostaa kontakteja muiden solujen ja ympäristönsä kanssa. Integriineillä on tärkeä rooli solun liikkumisessa sekä signalointiin liittyvissä toiminnoissa, ja syövässä integriinien toiminnan säätely muuttuu usein ihmiselle epäedulliseksi. Integriinien toimintaa tarvitaan myös solunjakautumisessa, ja jakautumissa tapahtuvien virheiden epäillään johtavan perimältään epästabiilien ja pahanlaatuisiin solujen syntyyn. Lisäksi tiedetään, että virheet integriinien kuljetuksessa ja muutokset integriinien ilmentymisessä vaikuttavat syövän invaasiokykyyn. Integriinien kuljetuksen merkityksestä syövän syntymisessä tai integriinien ilmentymisen lisääntymisestä onkogeenisäädellyssä invaasiossa on kuitenkin vain vähän aiempaa tutkimustietoa. Tässä väitöskirjassa tutkittiin integriinien roolia juuri näistä näkökulmista.

Useimmat ihmisen syöpäsolut ovat kromosomistoltaan poikkeavia, mutta edelleen on epäselvää onko tämä epänormaali perimä syövän syy vai seuraus. Häiritsemällä integriinien solunsisäistä kuljetusta terveissä soluissa olemme pystyneet osoittamaan, että tämä yksin on riittävä mekanismi solujen pahanlaatuiselle muuntumiselle. Nämä solut pystyvät muun muassa invasoimaan *in vitro* ja niistä muodostuu kasvaimia koeeläimissä. Näiden solujen perimässä tapahtuu järjestelmällisiä muutoksia, mm. tiettyjen geenien yli-ilmentymistä, joka johtaa niiden kontrolloimattomaan kasvuun. Transkriptiotekijä Twist2 ja Src kinaasi ovat osoittautuneet tärkeiksi tekijöiksi solujen pahanlaatuiselle muuntumiselle.

Etäpesäkkeiden muodostuminen on syöpäpotilaiden suurin kuolinsyy, minkä vuoksi syövän leviämistä säätelevät mekanismit ovat merkittäviä kohteita syöpäterapiassa. Integriinit säätelevät yhdessä syndekaani-perheen tarttumisreseptorien kanssa solun kiinnittymistä soluväliaineeseen. Tässä tutkimuksessa osoitettiin, että syndekaanit myös toimivat ko-reseptorina integriinivälitteisessä soluväliaineen mekaanisessa muokkauksessa. Lisäksi näytettiin että integriinit, syndekaanit ja matriksin metalloproteinaasit ovat välttämättömiä rintasyöpäsolujen invaasiossa ja, että K-ras-onkogeeni säätelee näiden molekyylien ilmentymistä.

Yhdessä nämä tulokset lisäävät tietoamme syövässä tapahtuvista monimutkaisista muutoksista ja syöpäsolujen invaasion ja etäpesäkkeiden kannalta keskeisistä tekijöistä.

Avainsanat: syöpä, aneuploidia, invaasio, integriini, syndekaani, Twist2, Src

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ABBREVIATIONS

2D, 3D	Two-dimensional, three-dimensional
aCGH	array Comparative genomic hybridization
ADAM	A disintegrin and metalloproteinase with thrombospondin motifs
Arp2/3	Actin-related protein 2/3
bHLH	basic Helix-Loop-Helix
CAF	Cancer-associated fibroblast
CAT	Collective to amoeboid transition
CDK	Cyclin-dependent kinase
CHO	Chinese hamster ovary
CIN	Chromosomal instability
CS	Chondroitin sulfate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DS	Dermatan sulfate
DUSP4	Dual-specificity phosphatase 4
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
EPS8	Epidermal growth factor receptor substrate 8
ERK	Extracellular signal-regulated kinase
FA	Focal adhesion
FACS	Fluorescence-activated cell sorting
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FERM	4.1 ezrin radixin moesin
GAG	Glycosaminoglycan
GAP	GTPase actvating protein
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GFR	Growth factor receptor
GTP	Guanosine triphosphate
HA	Hyaluronan
HAS1	Hyaluronan synthase 1
HGF	Hepatocyte growth factor
HMEC	Human mammary epithelial cell
HS	Heparan sulfate
IGF-1	Insulin-like growth factor-1
ILK	Integrin-linked kinase
IPA	Ingenuity pathway analysis
ITG	Integrin

JNK	c-Jun N-terminal kinase
KS	Keratan sulfate
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MAT	Mesenchymal to amoeboid transition
MEF	Mouse embryonic fibroblast
mFISH	multi-color Fluorescence in situ hybridization
MMP	Matrix metalloproteinase
MT1-MMP	Membrane type-1 MMP
PCR	Polymerase chain reaction
PDGF	Plateled-derived growth factor
PDZ	Postsynaptic density protein of 95, Discs large, Zonula occcludens 1
PG	Proteoglycan
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLK	Polo-like kinase
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homologue
RB	Retinoblastoma protein
RCP	Rab-coupling protein
RGD	Arginine-glycine-aspartic acid
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK	Rho kinase
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription PCR
SAC	Spindle assembly checkpoint
SDC	Syndecan
SFK	Src family kinase
siRNA	small-interfering RNA
SIRP-α	Signal-regulatory protein alpha
SCC	Squamous cell carcinoma
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I-III:

I **Högnäs, G**.*, Tuomi, S.*, Veltel, S., Mattila, E., Murumägi, A., Edgren, H., Kallioniemi, O. and Ivaska, J. (2012). Cytokinesis failure due to derailed integrin traffic induces aneuploidy and oncogenic transformation in vitro and in vivo. *Oncogene* 31. 3597-606 *Equal contribution

II **Högnäs, G**., Tuomi, S., Mattila, E., Laine, J.O., Vilkki, V., Murumägi, A., Edgren, H., Kallioniemi, O. and Ivaska, J. (2013). Aneuploidy facilitates oncogenic transformation via specific genetic alterations, including Twist2 upregulation. Submitted.

III Vuoriluoto, K., **Högnäs, G**., Meller, P., Lehti, K., and Ivaska, J. (2011). Syndecan-1 and -4 differentially regulate oncogenic K-ras dependent cell invasion into collagen through alpha2beta1 integrin and MT1-MMP. *Matrix Biol*. 30:207-17

 In addition, unpublished data are presented in this thesis.

INTRODUCTION

The process of tumorigenesis involves several steps that ultimately lead to conversion of a normal cell to a cancer cell. Cancer cells are characterized by a number of abilities, or hallmarks, that distinguish them from normal cells. In contrast to healthy cells, which respond to outside cues in a manner which is beneficial for the whole organism, cancer cells are in many regards oblivious to their environment and ignore signals that order them to stop growing or commit suicide. This can lead to excessive proliferation and formation of a local tumor. However, the most vicious aspect of cancer is the cells' ability to escape from the primary tumor and settle down in foreign tissues, the process of metastasis (Chambers et al., 2002). Metastatic growths are challenging therapeutic targets as they can be difficult to detect or dormant during decades, only to rapidly resume proliferation and develop into macroscopic tumors.

Cancer cells are also often aneuploid, that is they contain an abnormal number of chromosomes. Whether this property is a mere consequence of malignant transformation or actually contributes to tumorigenesis has been eluding scientists for decades. This is partly because of the difficulty in determining the sole influence of aneuploidy on tumor development and because aneuploidy as such is inherently growth inhibiting (Williams and Amon, 2009). Aneuploidy is also associated with chromosome instability, which could enable cancer cells to rapidly adapt to new environments via constant reshuffling of chromosomes. The existence of aneuploidy in cancer cells is thought to be the result of cell division failure that has subsequently led to generation of chromosomally aberrant progeny. Defective endo- and exocytic trafficking of membrane-bound receptors is a typical feature of malignant cells. As trafficking of the cell adhesion receptors integrins have been shown to be necessary for completing cell division, defective integrin traffic may have a causal role in the formation of aneuploid tumors.

Adhesion receptors on the surface of cells are also important in interpreting signaling cues from the environment and controlling cell motility. The integrin receptors consist of α and β subunit that bind to the extracellular matrix on the outside of the cell, and transmit signals to the inside of the cell that lead to reorganization of the actin cytoskeleton (Hynes, 2002). Similarly to integrins, the syndecan family of cell surface heparan sulfate proteoglycans bind to different matrix molecules which influences adhesive properties as well as cell signaling pathways (Tkachenko et al., 2005). Syndecan function is essential for development and tissue homeostasis and syndecans have also been shown to modulate integrin-mediated adhesion and signaling. Knowledge of the extent of cooperation between integrins and syndecans in 3D environments mimicking *in vivo* conditions is, however, still scarce. This cooperation is of particular interest in the invasive process and a deeper understanding would facilitate drug design and cancer therapy. In this thesis we have studied the role of integrin traffic in tumorigenesis and integrin expression and function in breast cancer invasion.

REVIEW OF THE LITERATURE

1. CANCER

Cancer is a common name for a group of diseases where uncontrolled cell growth has taken place in tissues of the body. The mechanisms that normally prevent cells from excessive proliferation have somehow been rendered dysfunctional in cancer cells and this can lead to the formation of a primary tumor. The capacity to invade and metastasize is what distinguishes a malignant tumor from a benign one and it is the most dangerous aspect of cancer; metastasis is the cause of 90% of deaths from cancer (Chambers et al., 2002). Cancer types are usually classified according to the tissue of origin and the level of malignancy. The majority of life-threatening, aggressive cancers occur in epithelial tissues and give rise to what is termed carcinomas. Less common forms of cancers are sarcomas stemming from cells of connective tissues and leukemia that originate in the immune system. Both genetic predisposition and environmental factors contribute to tumorigenesis, but the importance of different influences on cancer formation is largely dependent on the particular type of cancer in question. Most of the known carcinogens are also mutagens that induce mutations in DNA, resulting in activation of growth-promoting oncogenes, or inactivation of growth-inhibiting tumor suppressor genes. Activation of oncogenes can be the result of e.g. chromosomal translocations, gene amplifications or point mutations in the gene. Activation of only one allele of an oncogene is generally enough to confer a selective advantage to the cell. For tumor suppressor genes on the other hand, inactivation of both alleles is usually required and this can be achieved by deletions, insertions or epigenetic silencing of the gene (Luo et al., 2009).

1.1 Cancer progression and the malignant phenotype

Cancer development is a long process and many changes need to occur in a cell in order for it to develop into a fully transformed malignant cell. Many barriers have to be breached during the course of tumor progression, but cancer cells use different strategies to reach the endpoint of total malignancy. This is evident from the fact that a high degree of heterogeneity is usually seen in different cells from the same tumor and in the same tumor types derived from different individuals. Disruption of many different signaling pathways may lead to the same phenotype in different cancer cells. On the other hand, deregulation of a specific pathway in one cell type may not result in the same phenotypic changes in another cell type (Hanahan and Weinberg, 2000; Massague, 2004). In addition, the chronological order of events leading to full-blown malignancy also varies between cancer types, which makes it difficult to predict the multi-step process of tumorigenesis. In addition to invasive and metastatic abilities, among the altered properties characteristic for cancer cells are self-sufficiency in growth and proliferation signals, evading growth suppressors, resisting apoptosis, limitless replication, altered energy metabolism, genome instability, avoiding immune

destruction and induction of angiogenesis (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Kroemer and Pouyssegur, 2008; Luo et al., 2009). Some of these properties are considered a prerequisite for transformation, while others are associated with malignancy and could contribute to cancer progression in several ways.

1.1.1 Self-sufficiency in growth and proliferation

Different classes of growth signals transmitted through transmembrane receptors are required for normal cells to proliferate and grow. Anti-growth signals are likewise required to regulate growth. In tumor cells, however, deregulation of both of these control mechanisms leads to increased and uncontrolled proliferation. Anti-proliferation signals usually force cells into a reversible quiescent G0 state or into a postmitotic state that is accompanied by permanent differentiation. These signals consequently mediate their function by affecting the cell cycle clock, specifically the retinoblastoma protein (Rb). Rb binds to and inhibits E2F transcription factors from inducing expression of genes needed for cell cycle progression from G1 to S phase (Massague, 2004). In the majority of human cancers the Rb pathway is disrupted, for example by activation of cyclin-dependent kinase 4 (Cdk4) or cyclin D1, important for phoshorylation and inactivation of Rb, or inactivation of the Cdk4 inhibitor $p16^{Ink4a}$ or Rb itself (Liu et al., 2004; Polager and Ginsberg, 2009). The tumor suppressor p53 is important in activating cell death, but also functions to halt cell cycle progression by inducing expression of the Cdk inhibitor $p21^{Cip1}$. In the majority of epithelial tumors, both the p53 and Rb pathways have been disrupted, demonstrating the importance of these two proteins as guardians against out-of-control cell growth (Polager and Ginsberg, 2009).

Cancer cells often find ways to activate stimulatory pathways in the absence of stimuli from the cell exterior. Signaling through receptor tyrosine kinase (RTK) pathways for example often lead to transcription of growth-promoting genes and many genes involved in these pathways have been shown to be altered in cancer (Takeuchi and Ito, 2011). The upstream receptors themselves, such as epidermal growth factor receptor (EGFR), c-Met or Her2 are often overexpressed in cancers and tumor cells can also make their own mitogens. For example, in a human bladder carcinoma cell line, the c-Met receptor is phosphorylated under serum-free conditions. An autocrine signaling loop involving EGFR ligands, EGFR, Src kinase and c-Met is thought to be involved in the survival of the cells, because abrogation of these proteins results in cell death in response to serum-deprivation (Yamamoto et al., 2006). One important pathway downstream of many RTKs is the Ras-MAP-kinase pathway and in many human cancers Ras proteins are structurally modified so that they signal continuously (Bos, 1989). The cell adhesion receptors integrins often regulate the same pathways as growth factor receptors and crosstalk between these two receptor types is used to stimulate proliferation (Ivaska and Heino, 2011; Khwaja et al., 1997; Miyamoto et al., 1996). For example, the Src family kinase (SFK) Fyn is activated upon integrin-extracelullar matrix binding and binds to and phosphorylates the adaptor protein Shc. This creates a binding site for the Grb2/Sos complex that activates the Ras-MAP kinase pathway (Wary et al., 1998). Grb2/Sos can also be recruited to focal adhesions

via binding to focal adhesion kinase (FAK) (Schlaepfer et al., 1994). Integrin-mediated adhesion to the extracellular matrix (ECM) is usually required for normal cells to progress through the cell cycle. Expression levels of cyclin D1, phosphorylation of Rb and activity of cyclin E-Cdk2 is adhesion-dependent (Zhu et al., 1996). Also, the c-Jun NH_2-terminal kinase (JNK) regulates cyclin D1 and progression through G1; JNK activation requires integrin ligation and the association of FAK with Src and $p130^{CAS}$ (Ip and Davis, 1998). The actin cytoskeleton, whose regulation is largely integrin-dependent, also controls cell cycle progression. Integrin ligation and activation of the actin regulators Cdc42 and Rac1 has been shown to induce proteasomal degradation of the Cdk inhibitor $p21^{Cip1}$ in different cell types (Bao et al., 2002).

Phosphatidylinositol 3-kinases (PI3Ks) are a family of proteins activated through many different signaling agents. PI3Ks phosphorylate phosphatidyl inositol phosphate lipids to generate phosphatidylinositol 3,4,5 trisphosphate (PIP_3), which in turn creates docking sites for proteins that carry domains able to bind to the phoshorylated inositol head group. A major signaling pathway downstream of PI3K is mediated via Akt kinase whose activation ultimately leads to increased proliferation and cell growth by inactivating GSK-3β, reducing the levels of $p21^{Cip1}$ and $p27^{Kip1}$ and activating mTOR (Vivanco and Sawyers, 2002). The PI3K pathway is deregulated in many types of cancer, often because of decreased activity of the PIP_3 phosphatase PTEN, which is frequently mutated or lost in human cancers. Activating mutations in the catalytic subunit p110α of PI3K are also common in solid tumors, while increased levels of p110δ have been detected in leukemia (Lee et al., 2005; Samuels et al., 2004; Sujobert et al., 2005). The Akt pathway is also closely involved in regulating energy metabolism. To support increased proliferation associated with the malignant state, tumor cells often display metabolic alterations. Increased glucose uptake and lactate production indicates high utilization of the glycolytic pathway. Glycolysis normally takes place when oxygen levels are low, but cancer cells process glucose via this pathway even in conditions of high oxygen. Although glycolysis alone produces less energy than using oxidative phosphorylation in mitochondria, it is thought that acidification of the microenvironment that follows lactate production promotes tumor invasion and that glycolytic intermediates are more beneficial for the cancer cell as they can be shunted into various anabolic pathways (DeBerardinis et al., 2007; Hatzivassiliou et al., 2005; Martinez-Zaguilan et al., 1996; Vander Heiden et al., 2009). Metabolic reprogramming thus favors cancer cell growth through increased biosynthesis of amino acids, nucleotides and lipids. These alterations could also be taken advantage of therapeutically, as cancer cells become dependent on alternative metabolic pathways for their survival due to their increased energy needs.

1.1.2 Unlimited replication

Mammalian cells also have an intrinsic program that limits their multiplication to a certain number of division cycles. A cancerous growth has to overcome this replication barrier in order to develop into a macroscopic tumor. A cell that can proliferate indefinitely is said to be immortal and most tumor cells growing in culture seem to have this ability, which suggests that unlimited replicative potential has been a

necessary acquisition during tumor progression. In cultured cells, the loss of the ability to divide is termed replicative senescence. Senescence is an irreversible state of growth arrest and can be induced by different forms of stress, and especially successive shortening of telomeres. Telomeres are DNA repeats at the ends of chromosomes, which protect the chromosomes from degradation and from end-to-end fusion (Greider and Blackburn, 1996). Senescence can be overcome in human fibroblasts by the abrogation of the tumor suppressor proteins p53 and Rb (Shay et al., 1991). Overcoming senescence is, however, not enough for immortalization of human cells. Inactivation of checkpoint responses allows replication to continue until cells eventually encounter another proliferative barrier, called cellular crisis, due to additional telomere shortening. This ultimately leads to chromosomal fusions causing karyotypic chaos, large-scale apoptosis and crisis (Maser and DePinho, 2002). Many cancer cells have found a way to enable unlimited replication and protect their DNA by expression of telomerase, which adds telomeric repeats onto the ends of DNA. Most human cells do not express telomerase and it is thought that crisis, while leading to the death of most cells in a population, would produce a rare immortal cell which has acquired telomerase activity and extensive cancer-associated genomic changes (Artandi et al., 2000; Counter et al., 1992). Thus, telomere shortening may both restrict and promote malignant transformation (Hanahan, 2000; Maser and DePinho, 2002).

1.1.3 Evasion of apoptosis

Normally the number of cells in a population is determined and maintained not only by regulation of cell proliferation but also by apoptosis, i.e. programmed cell death. External death signals are mediated by among others the Fas ligand (FasL) and tumor necrosis factor α (TNF-α) which bind to their respective receptors (Schulze-Osthoff et al., 1998). This signaling initiates a caspase cascade that leads to destruction of subcellular structures, organelles and the genome. DNA damage, activation of oncogenes, shortage of oxygen or lack of survival factors can also induce the apoptotic program (Giancotti and Ruoslahti, 1999; Green and Evan, 2002). One of the most important regulators of apoptosis is the transcription factor p53. Upon cellular stresses p53 induces expression of pro-apoptotic proteins and initiates the apoptotic cascade by releasing cytochrome c from mitochondria.

In a normal cell, ECM detachment does not only cause cell cycle arrest, but also induces a form of programmed cell death termed anoikis. Anoikis is a specific form of caspase-mediated apoptosis induced by inappropriate or insufficient integrin-ECM interactions and it serves to maintain tissue integrity and prevents cells from surviving in incorrect locations and thus also tumor cell dissemination (Frisch and Screaton, 2001). FAK is a key factor in protecting cells from anoikis by binding to PI3K via its autophosphorylation site and activating Akt-dependent survival signals (Chen et al., 1996). Akt can for example phosphorylate and inactivate the pro-apoptotic proteins Bad and caspase 9 (Cardone et al., 1998; Datta et al., 1997; Vivanco and Sawyers, 2002). Furthermore, integrins and EGFR together regulate the expression of pro-apoptotic Bim, which is induced in many epithelial cell lines upon detachment. EGFR expression was shown to be dependent on β1 integrin signaling and overexpression of

EGFR or the Erk kinase pathway in suspended cells resulted in reduced Bim levels and protection from anoikis (Reginato et al., 2003). Absence of attachment thus normally leads to both cell-cycle inhibitory signals as well as induction of apoptotic pathways. Cancer cells circumvent these control systems by hyperactivation of mitogenic signaling, by activation of survival factors or by changing the surface expression of integrins and thereby adapting to new environments. These changes consequently enable anchorage-independent growth, a hallmark of malignancy (Frisch and Screaton, 2001). This property is necessary for cancer cells to be able to survive and spread in the lymph or blood vessels and settle down into foreign microenvironments.

1.1.4 Genetic instability

Most alterations that need to happen for cell transformation to occur are due to changes of the genome that usually involve mutations. However, DNA repair mechanisms and other processes governing genomic integrity keep the rate of mutations at a relatively low level. In order for cancer to have time to evolve during a human life span, cells presumably need to acquire increased mutability (Loeb, 2001)**.** Some genetic hits, such as mutations in genes controlling genomic integrity, enhance the mutation rate which in turn promotes further lesions. This results in an increased probability of producing a cell with a higher proliferation rate that can undergo clonal selection and overcome selection barriers (Cahill et al., 1999; Vogelstein and Kinzler, 2004). However, it has also been argued that too much instability or excessive oncogenic signals may in fact evoke cell death (Birkbak et al., 2011; Cahill et al., 1999; Weaver et al., 2007; Weaver and Cleveland, 2009). A form of instability called chromosomal instability (CIN) is frequently detected in cancer and is thought to be a major contributor to tumor formation. CIN is characterized by recurrent missegregation of chromosomes during multiple divisions and thus provides continuous gains, losses and rearrangements of chromosomes. Cells with selective advantages are thus produced with higher frequency (Chandhok and Pellman, 2009; Geigl et al., 2008). CIN induced by the mitotic checkpoint protein Mad2 has also been shown to increase recurrence of lung tumors in which the driving mutation has been targeted (Sotillo et al., 2010). CIN can thus facilitate adaptation and transformation even after oncogenic withdrawal. The influence of CIN on tumor formation and whether it favors or suppresses it is nevertheless very much dictated by the particular context and the rate of instability (Komarova and Wodarz, 2004; Kops et al., 2004).

2. CELL ADHESION AND THE EXTRACELLULAR MATRIX

Extracellular matrix (ECM) is present in all tissues and organs of the body. It is important in providing strength and elasticity to organs and protects them by maintaining water retention and extracellular homeostasis. The ECM supports tissue integrity, functions as a barrier and an anchorage site, provides tracks for the movement of cells, supports signaling by binding and presenting growth factors to their respective receptors and possesses biomechanical properties that influences cell behavior (Lu et al., 2011). The ECM mainly consists of proteoglycans, fibrous proteins and water and cells interact

with the ECM via membrane-bound adhesion receptors such as integrins and syndecans that bind to different matrix components. Proteoglycans (PGs) are composed of a protein core that is covalently linked to a glycosaminoglycan (GAG) side chain. GAGs are negatively charged heteropolysaccharides composed of repeating disaccharide units and differences in sulfation generates chondroitin sulfate (CS), heparan sulfate (HS), dermatan sulfate (DS) and keratan sulfate (KS) GAGs (Afratis et al., 2012). Hyaluronan is the only GAG that lacks sulfation as well as a core protein. PGs such as perlecan, lumican, aggrecan, decorin and hyaluronan form hydrophilic gels that are very force-resistant. The main fibrous proteins in the ECM are collagens, fibronectins, laminins, elastins and tenascins.

The exact composition of the ECM varies between organs and tissues. In tendons, bone, cartilage and skin triple helical collagens form fibril bundles that provide tensile strength. Fibrillar collagens are the most abundant proteins in interstitial matrix, whereas the basement membrane contains collagen IV that forms three-dimensional networks (Kadler et al., 2007). The basement membrane, a ~100 nm specialized ECM layer that separates endothelium and epithelium cell monolayers from the underlying connective tissue, also contains laminin, nidogen and perlecan (LeBleu et al., 2007). The ECM is constantly being remodeled to suit the needs of the tissues and matrix remodeling is especially important in developmental processes such as branching morphogenesis, tooth and skeletal development, angiogenesis and in maintaining stem cell niches in different organs. Cells likewise respond to differences in biomechanical properties of the matrix and the interaction between cells and the ECM is therefore highly dynamic. Defects in the processes regulating ECM dynamics can consequently be devastating for tissue homeostasis and contribute to a wide range of disorders, including cancer.

2.1 Cancer cell invasion

What makes cancer such a deadly disease is the ability of cancer cells to metastasize, i.e. escape from the primary tumor and move to distant sites in the body where they form new malignant growths. Epithelial cell cancers can acquire the ability to break through the underlying basement membrane and invade the stroma, where tumor cells have better access to the oxygen and nutrients supplied by the nearby blood and lymphatic vessels. The malignant cells intravasate into the lumina of these vessels, which provide transportation routes for the tumor cells to remote areas of the body. Once the tumor cells have escaped from the vessel and extravasated into the tissue parenchyma, colonization, the formation of a new macroscopic tumor, can take place (Chambers et al., 2002). This is usually the most difficult and rate-limiting step in tumorigenesis, largely because the new location may differ considerably from the tissue in which the tumor originated. The new surroundings have to provide the tumor with adequate growth factors needed for its survival and be permissive for the formation of macroscopic tumors. Cancer cells are thus very much influenced by the environment because it can restrict growth but also actively aid in the formation of secondary tumors. For example, stromal cells are often co-opted into releasing proliferation and survival signals and aiding in induction of invasion, angiogenesis, inflammation and ECM degradation and remodeling. They

provide signals such as transforming growth factor β (TGF-β), tumor necrosis factor α (TNF-α), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and matrix metalloproteinases (MMPs) to the invasive front of the primary tumor (Fukumura et al., 1998; Mueller and Fusenig, 2004; Wyckoff et al., 2004). Various combinations of these signals can initiate transformed cells to become invasive.

2.1.1 Regulation of cell motility and invasion

The molecular pathways regulating invasion and motility as well as the specific mode of invasion employed by a cancer cell is largely cell-type specific in addition to the influence of the microenvironment. Modulation of cell-cell contacts as well as cell-matrix interactions is necessary for cancer cells to acquire a more migratory phenotype. The morphology of individual cells invading through a matrix can vary greatly -some cells assume a rounded, amoeboid cell shape, while others invade in a more elongated, mesenchymal manner (Figure 1). In a process called epithelial-mesenchymal transition (EMT), carcinoma cells assume a shape and gene expression profile resembling that of mesenchymal cells, such as highly motile fibroblasts (Thiery, 2002). This is a process normally occurring in embryogenesis and wound healing. Snail, Slug and Twist are examples of transcription factors that function during embryogenesis to convert epithelial cells to migratory mesenchymal cells and they are therefore essential for morphogenetic processes such as development of the heart and formation of the neural crest (Chen and Behringer, 1995; Nieto et al., 1994; Sefton et al., 1998). These transcription factors have also been shown to repress transcription of the E-cadherin gene, encoding an adhesion molecule important in the structure and strength of the epithelial sheets, and loss of E-cadherin has been detected in a variety of invasive cancers (Batlle et al., 2000; Bolos et al., 2003; Yang et al., 2004). Cancer cells that have undergone EMT can break free from the tumor mass and invade as individual cells. However, many cancer types including breast, prostate and lung tumors, rhabdomyosarcoma and melanoma, maintain cell-cell cohesive structures and may thus penetrate vessel walls collectively as cell strands (Ewald et al., 2012; Hegerfeldt et al., 2002; Parri and Chiarugi, 2010; Wolf et al., 2007).

2.1.1.1 Rho GTPases, kinases and related molecules

The morphology and motility of cells are largely regulated by the Rho family of monomeric GTPases Cdc42, Rac and RhoA. These GTPases act as molecular switches that cycle between an active GTP-bound form and an inactive GDP-bound form. They regulate cell morphology and the actin cytoskeleton and also control survival, proliferation and gene expression (Hall, 1998; Sahai et al., 2001; Sahai and Marshall, 2002). Generally, activation of RhoA in adhered cells leads to formation of contractile actin-myosin filaments called stress fibers and focal adhesions, which are specialized sites of attachment between the actin and the ECM consisting of large, dynamic protein complexes. Rac induces the formation of wide and flat, sheet-like protrusion structures called lamellipodia that contain a cross-linked network of actin filaments, while activation of Cdc42 leads to formation of filopodia (Hall, 1998). Filopodia are

protruding structures within the lamellipodia that contain long, bundled actin filaments. Filopodia are thought to sense tactic signals and establish the direction of movement.

Localized activation and deactivation of these GTPases are spatially and temporally controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), by different lipid modifications and tissue specific effectors. This regulation ensures motility, but the role of GTPases in migration on two-dimensional (2D) or invasion into three-dimensional (3D) matrixes may be very different. One example is the RhoA-Rho kinase (ROCK) signaling pathway. ROCK is one of the downstream effectors of RhoA and ROCK-mediated phosphorylation of myosin light chain leads to crosslinking of actin filaments and generation of contraction. In colon carcinoma cell lines invading with an elongated morphology inhibition of RhoA or ROCK have no effect on the cells' ability to invade into the 3D matrixes Matrigel or collagen I. In contrast, the same treatment significantly impaired the ability of another colon carcinoma cell line and a melanoma cell line with round morphology to invade (Sahai and Marshall, 2003). RhoA and ROCK signaling are thus necessary for rounded cell motility (Figure 1), but this is specific for 3D matrix as migration is not blocked in these cells on 2D surfaces. RhoA has been shown to influence the localization of ezrin (Yonemura et al., 2002) and similarly to blocking RhoA, inhibiting ezrin function impaired invasion only in round cells. These and other studies have shown that cells invading in an amoeboid fashion need RhoA to induce a high level of actomyosin contractility to be able to squeeze through holes in the matrix (Figure 1) (Sahai and Marshall, 2003; Sanz-Moreno et al., 2011; Wolf et al., 2007; Wyckoff et al., 2006).

In contrast to amoeboid motility, cells invading in an elongated mode are usually dependent on MMP -mediated degradation of the ECM and have stronger cell-ECM adhesions. Typically, Rac1 activation at the leading edge leads to protrusion formation and directionality which is maintained by Cdc42. Integrin-dependent focal complexes are formed at the front of the cell that can mature into focal adhesions. RhoA activity is usually low at the leading edge (partly due to Rac-1-mediated inhibition) and higher at the back where RhoA promotes actomyosin contraction and, together with disassembly of adhesions, detachment of the trailing edge (Friedl and Wolf, 2003; Sanz-Moreno et al., 2008). Blocking RhoA function in mesenchymal-type cells may therefore have little effect on motility (Sahai and Marshall, 2003). The leading cell of collectively invading HT1080 fibrosarcoma cells and MDA-MB-231 breast carcinoma cells, have also been shown to use membrane type-1 matrix metalloproteinase (MT1-MMP)-mediated collagen degradation and β1-integrin-ECM adhesions to move forward. In these cells, adherens junctions are maintained to keep the cell cluster together (Wolf et al., 2007). Cells that migrate as clusters often behave in a cooperative manner where force and contractility is mediated via cell-cell junctions making the cohort behave as one big cell (Friedl and Alexander, 2011; Hidalgo-Carcedo et al., 2011). In collectively invading squamous cell carcinomas (SCCs) actomyosin function has been found to be higher around the edge of cell clusters in regions that are in contact with the ECM (Gaggioli et al., 2007). Disruption of cortical myosin light chain localization and phosphorylation via knockdown of Cdc42 and its effector MRCK also inhibited cell invasion (Gaggioli et al.,

2007). At the cell-cell junctions actin fibers stabilize E-cadherin, but excess adhesion between cells can also block collective migration (Omelchenko, 2012). Actomyosin contractility is thus tightly regulated at cell-cell contacts in collectively migrating cells. Silencing of RhoA inhibits the formation of cell-cell contacts while RhoA overexpression, depletion of the RhoGAPs myosin IXA or p190RhoGAP or depletion of RhoE, which antagonizes ROCK-driven actomyosin contractility, lead to cell scattering and impaired collective migration of different epithelial cells (Desai et al., 2004; Hidalgo-Carcedo et al., 2011; Omelchenko and Hall, 2012). Mechanisms regulating collective cell migration have also been elucidated by studying different developmental processes, where this type of migration is common, as well as cancer cells. Rac activation in *Drosophila* border cells has for instance been shown to direct the migration of the whole cluster of cells (Inaki et al., 2012). This study showed that one cell with overall higher Rac activity guided the migrational direction of the cluster and that localized activation within this cell was less important (Inaki et al., 2012).

Especially mesenchymal-type cells often rely on signals mediated by Src tyrosine kinase to acquire increase motility. Src is activated downstream of both RTKs and integrin adhesion receptors. It is a very potent oncogene with a variety of cellular substrates that elicit both transcriptional responses and regulate cell shape. For this reason, Src is able to induce both increased proliferation and survival of cancer cells, as well as promote motility and invasion. Together with FAK, Src affects Rho GTPase signaling by modulating GEF and GAP signaling downstream of integrins. For example, Src activates Rac1 at the leading edge by promoting activation of the GEF DOCK180 via recruitment of adaptor proteins Cas and Crk (Chodniewicz and Klemke, 2004), via phosphorylation and activation of the Rac GEF Tiam1 or by phosphorylating Syk which subsequently activates the Rac GEF Vav1 (Obergfell et al., 2002; Servitja et al., 2003). Src is also able to transiently suppress RhoA activity at the front of the cell by phosphorylation and activation of p190RhoGAP (Arthur et al., 2000). However, formation of podosomes, adhesive structures containing proteolytic enzymes, has also been shown to require Src-mediated activation of RhoA (Berdeaux et al., 2004). Src or FAK null cells show reduced migration and also have enlarged and stronger cell-matrix adhesions. Src/FAK signaling is therefore thought to promote motility by increasing focal adhesion turnover (Ezratty et al., 2005; Fincham and Frame, 1998; Webb et al., 2004). In addition, Src has been shown to induce expression of several MMPs. In fibroblasts, Src/FAK-mediated activation of Rac1 and JNK upregulates MMP9 levels and activates MMP2, resulting in enhanced invasion in Matrigel (Hsia et al., 2003). Src can also induce EMT by deregulating E-cadherin, for example by disruption of its localization in KM12C colon cancer cells or inducing endocytosis of the E-cadherin complex in epithelial cells (Avizienyte et al., 2002; Fujita et al., 2002).

In line with these promigratory functions, there is frequently increased activity of the Rho family of monomeric GTPases and Src/FAK signaling in many types of cancer cells (Parri and Chiarugi, 2010). The Rho isoform RhoC is also important for contractility and motility and has been associated particularly with metastasis. For example in breast cancer cells, microRNA-10b inhibits expression of HOXD10 leading

to the subsequent upregulation of RhoC-mediated invasion. microRNA-10b was shown to be induced by the EMT-related transcription factor Twist (Ma et al., 2007). A difficult problem in targeting tumor cell invasion is the plasticity of the cancers, i.e. their ability to switch from one mode to the other. For example, inhibition of proteases or integrins have no effect on some mesenchymal-type colon carcinoma cells, fibrosarcomas and melanomas as they instead switch to an amoeboid shape (Sahai and Marshall, 2003; Sanz-Moreno et al., 2008; Wolf et al., 2003; Wolf et al., 2007). Indeed, cancer cells have been shown to undergo both mesenchymal to amoeboid transition (MAT), collective to amoeboid transition (CAT) as well as EMT. However, invasion of colon carcinoma cells undergoing MAT could at least be blocked by treating the cells with both protease inhibitors and the ROCK inhibitor Y27632 (Sahai and Marshall, 2003), suggesting that tumor plasticity could be targeted by simultaneous use of multiple approaches.

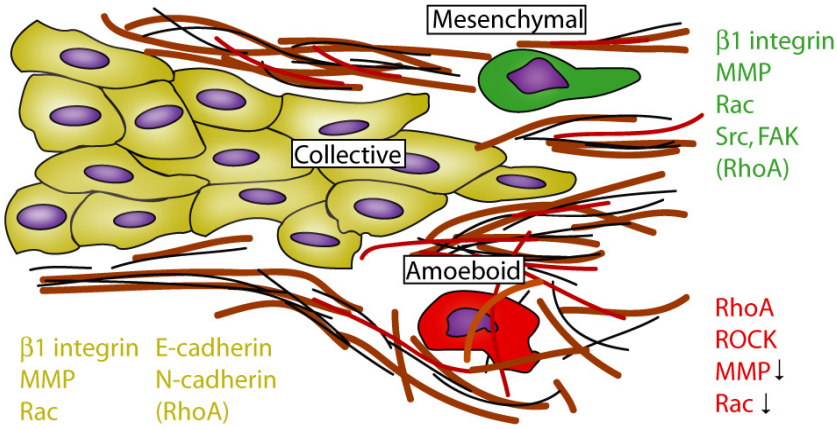

Figure 1. Different migration modes employed by cancer cells. Factors generally involved in the regulation of the different types of migration are indicated. After detachment from the cell mass, migration mode is partly dictated by the strength of adhesion and contractility and ability to remodel the ECM. Cells migrating in an amoeboid fashion do not degrade the ECM and instead rely on actomyosin contracility to squeeze through gaps in the matrix. See text for details. Adapted from Friedl and Alexander, 2011.

2.1.2 Matrix remodeling

To be able to invade, cancer cells need to push away obstacles and dig passageways through the ECM of the nearby tissues. The ECM is remodeled through rearrangements of matrix components by cleavage and deposition. For this purpose, cancer cells use different matrix-degrading proteases, secreted either by themselves or by stromal constituents like fibroblasts, macrophages and mast cells (Figure 2). The MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families are the main enzymes responsible for matrix degradation. Enzymes involved in remodeling are regulated at the transcriptional level and by ensuring proper localization at the cell surface. MMPs are also controlled by tissue inhibitors of metalloproteinases (TIMPs) and many MMPs are synthesized as inactive pro-enzymes that need to be activated by other

proteases, often other MMPs. In addition, MMPs can either be membrane-bound or elicit their function as secreted soluble proteins. Despite the tight control of MMP activity, these enzymes are frequently overexpressed and activated in malignant tissues (Kessenbrock et al., 2010). MMPs cleave collagens, proteoglycans, fibronectin, laminin and tenascin of the ECM to make way for the cells to move and they also mobilize previously inactive growth factors from the ECM to the tumor cells' benefit (Tatti et al., 2008) (Figure 2). Collagen I can be cleaved by the soluble MMP-1, MMP-8 and MMP-13 enzymes, but also by the transmembrane MMP14 (MT1-MMP). Especially MT1-MMP is necessary for collagenolytic activity and invasion in collagen in both sarcomas and carcinomas (Sabeh et al., 2004; Wolf et al., 2007). In addition to collagen I, MT1-MMP also degrades collagen II and III, fibronectin and laminin and is able to activate pro-MMPs. MT1-MMP was also shown to be needed for invasion of cancer cells through reconstituted basement membrane (Hotary et al., 2006) and MT1-MMP overexpression in normal epithelial cells leads to increased formation of locally invading tumors (Soulie et al., 2005).

Studying cell-matrix interactions *in vitro* has been done on both 2D surfaces where cells are adhering to a matrix coat and in 3D conditions where cells are embedded into the matrix. Differences in matrix stiffness and architecture can influence intracellular signaling pathways and examining these pathways under more *in vivo*-like 3D conditions is therefore becoming increasingly important. Examples of such assays are the Boyden chamber migration and invasion assays, gel contraction assays and organotypic models where a mixture of different matrix constituents and stromal cells are used. In floating collagen assays, fibroblasts embedded into the matrix adhere to the collagen fibers and contract the network to a denser structure. The ability of cells to exert mechanical force and contract the matrix reflects their ability to remodel it (Gaggioli et al., 2007). In fibroblasts, PDGF has been shown to induce matrix contraction via activation of Rho kinase (ROCK) and p21 activated kinase 1 (Pak1) (Abe et al., 2003; Rhee and Grinnell, 2006). In contrast, lysophosphatidic acid (LPA)-induced contraction was not dependent on ROCK, but instead induced co-operation of Pak1 with the Rho effector mDia1 (Rhee and Grinnell, 2006). Pak1 silencing led to reduced membrane ruffling and matrix contraction. Using function blocking antibodies or knock-out cells, it has also been shown that collagen binding integrins α2β1 and α11β1 are important for contraction of collagen I (Langholz et al., 1995; Svendsen et al., 2009). Some carcinomas have a limited ability to remodel the matrix and invade and instead hijack stromal fibroblasts to do the job for them (Figure 2). Cancer cell-derived growth factors and cytokines can promote integrin α5-mediated activation of ROCK, as well as activation of JAK1 and induction of pStat3 in stromal cells, thus converting them into so called cancer-associated fibroblasts (CAFs). These CAFs are able to contract collagen and also make passageways in the surrounding matrix by JAK1-ROCK- controlled acto-myosin contractility, thereby enabling cancer cell invasion (Cirri and Chiarugi, 2011; Gaggioli et al., 2007; Sanz-Moreno et al., 2011). A chemical screen identifying compounds that inhibit collagen gel contraction also revealed a role for the small GTPase Rab21 in this process. Rab21-mediated delivery of integrin α5 to the plasma membrane was needed for induction of RhoA-mediated contractility (Hooper et al., 2010).

In addition to just clearing tracks in the ECM, matrix remodeling can also facilitate migration of cancer cells by modifying the orientation of matrix fibers. In breast tumors, collagen I is often linearized rather than deposited as relaxed fibrils and this could facilitate carcinoma migration along the matrix (Levental et al., 2009; Wolf et al., 2007) (Figure 2). Cells likewise sense the stiffness of the matrix, which is to a large extent defined by its architecture. The force exerted by the matrix on the cells, and vice versa, is subsequently mediated to the interior of the cell and ultimately determines gene expression and cell behavior (Butcher et al., 2009; Zaman et al., 2006). Breast cancer tissue is often stiffer than normal tissue due to increased collagen deposition or crosslinking, or reduced turnover (Figure 2). (Butcher et al., 2009; Paszek et al., 2005). Increased collagen crosslinking can lead to increased integrin-mediated signaling and motility and is often due to excess activity of the enzyme lysyl-oxidase (LOX)(Baker et al., 2012; Levental et al., 2009).

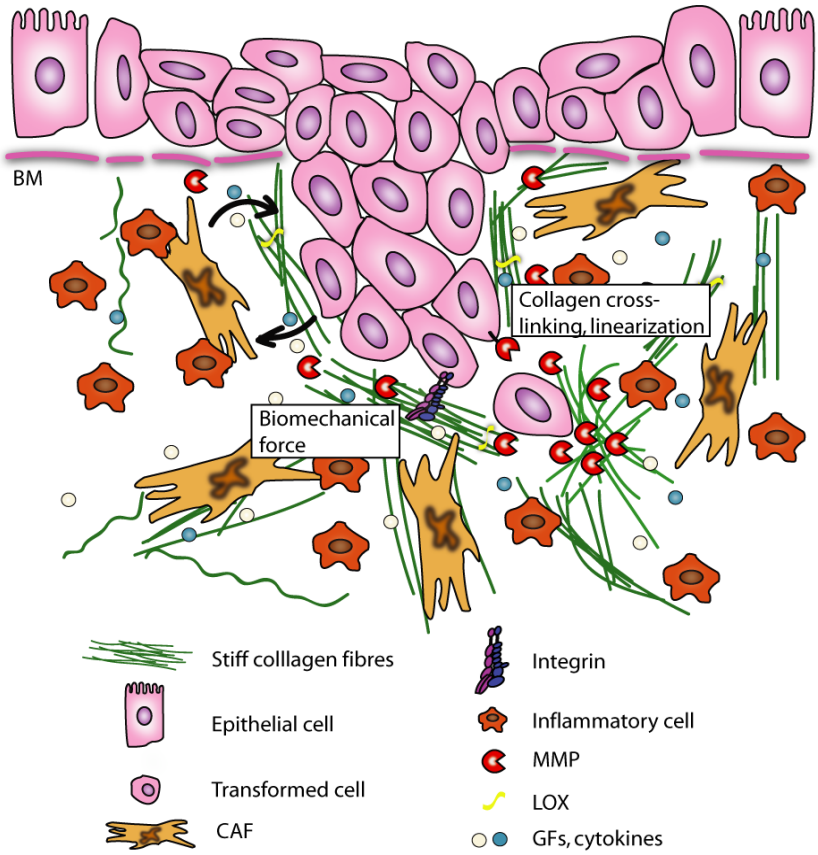

Figure 2. Interaction between cancer cells and the microenvironment. When cancer cells invade into the underlying stroma the ECM provides anchorage and tracks for cell migration and functions as a reservoir for growth factors. There is also extensive crosstalk between cancer cells and stromal cells: both release growth factors, cytokines, proteases and ECM molecules which enhance cancer cell invasion. The stiff, crosslinked matrix fibers influence cell behavior via cell adhesion receptors. BM, basement membrane; CAF, cancer-associated fibroblast; GF, growth factor; MMP, matrix-metalloproteinase; LOX, lysyl-oxidase. Adapted from Frantz et al., 2010.

2.2 Integrins

The integrins are a family of transmembrane cell-matrix adhesion receptors that function as the link between the ECM and the interior of the cell. Integrins are expressed in all multicellular organisms and regulate crucial cellular functions such as cell motility, proliferation and survival. Integrins are therefore indispensable factors in many physiological processes, including development, angiogenesis and immune responses (Hynes, 2002; Yang et al., 1993).

2.2.1 The integrin family

The integrin receptor is a heterodimer composed of non-covalently linked α and β glycoprotein subunits (Figure 3). In mammals there are 8 types of β subunits and 18 types of α subunits, which form heterodimers in 24 different combinations. Among the ligands of integrins are ECM proteins fibronectin, vitronectin, collagen and laminin, as well as cell-surface bound counter-receptors like intercellular adhesion molecules (ICAM) and vascular cell-adhesion molecules (VCAM). Ligand specificity is determined by the composition of the α and β subunits (Hynes, 2002) and integrins are usually categorized according to ligand binding specificity or evolutionary origin (Hynes, 2002; Johnson et al., 2009). α3β1, α6β1, α6β4 and α7β1 bind to laminin and are thus the major basement membrane receptors. α5β1, α8β1, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and αIIbβ3 form another evolutionarily related subgroup. These heterodimers recognize the short arginine-glycine-aspartic acid (RGD) motif present in fibronectin, vitronectin and the plasma protein fibrinogen. Collagen binding receptors are comprised of α1β1, α2β1, α10β1, α11β1 (White et al., 2004), while leukocyte integrins αEβ2, αLβ2, αMβ2, αDβ2 and αXβ2 make up a separate group. The two latter subgroups are also distinguished by the presence of an αI domain, which determines ligand recognition, in the alpha subunit (Johnson et al., 2009). In addition to these categories, α4β1, α4β7 and α9β1 integrins form their own group. These heterodimers bind ECM molecules and have also been shown to be important for angiogenesis by binding to VCAMs and vascular endothelial growth factors (Garmy-Susini et al., 2005; Vlahakis et al., 2007).

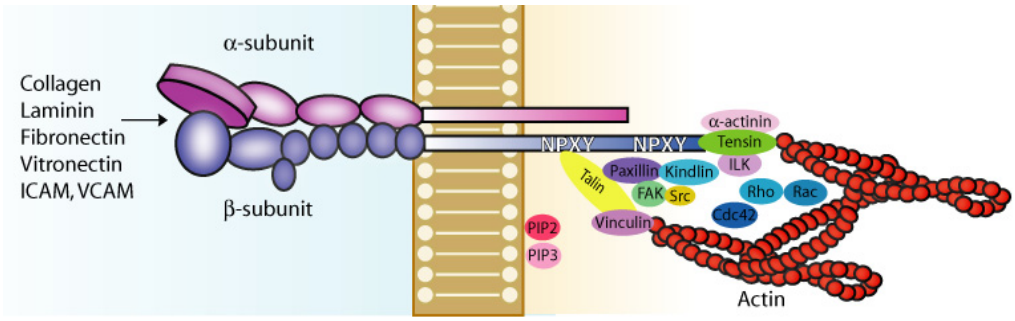

Figure 3. The integrin heterodimer. Integrins consist of α and β subunits and can be activated by proteins binding to the cytoplasmic domain and matrix ligand binding on the extracellular side. This leads to recruitment of focal adhesion proteins, intracellular signaling and reorganization of the cytoskeleton. See text for details. FAK, focal adhesion kinase; ILK, integrin-linked kinase; PIP_2, phosphatidylinositol 4,5 bisphosphate; PIP_3, phosphatidylinositol 3,4,5 trisphosphate. Adapted from Morgan et al., 2007.

2.2.2 Integrins in development and disease

The β1-integrin subunit, which forms heterodimers with a large number of α subunits, is important for many developmental process and β1 knock-out mice die early at E5 (Fassler and Meyer, 1995). Deficiency of the α-subunits α5, α4 and αv also causes embryonic lethality in mice due to among other things defective development of the heart, placenta and vasculature (Bader et al., 1998; Yang et al., 1993; Yang et al., 1995). Ablation of β2 leads to defects in leukocyte function while lack of many integrins causes skin phenotypes. For example the skin blistering disease epidermolysis bullosa in humans can be caused by mutations in integrin α6 or β4 (Pulkkinen and Uitto, 1999). Integrin αIIb or β3 mutations impairs platelet aggregation causing a bleeding disorder (Hogg and Bates, 2000). Apart from a mutation of the β1 subunit detected in squamous cell carcinoma (Evans et al., 2003), β1 integrin mutations are in general rare in cancer. Deregulated expression or increased ligand affinity are more common and have been detected in several different cancer types. For example, elevated expression of integrin α5β1, αvβ3 or α2β1 is associated with metastasis in melanoma, prostate carcinoma and breast carcinoma (Laidler et al., 2000; McCabe et al., 2007; Nip et al., 1992; Sloan et al., 2006). Function-blocking monoclonal antibodies or RGD peptide mimetics are currently used for targeting specific integrins in clinical trials. However, some cancers display a decrease in integrin expression (Zutter et al., 1995) and inhibiting one heterodimer can cause increased function of another, ultimately increasing cell motility (Caswell et al., 2008). Targeting integrins can therefore have unexpected consequences and is also complicated by the fact that normal cells rely on integrins for proper function.

2.2.3 Activation and signaling

The integrin heterodimer consists of large extracellular domains, transmembrane domains and short cytoplasmic tails and integrin activation can be achieved both on the extra- and intracellular side of the plasma membrane (Figure 3). Inactive integrins are thought to exist in a conformation where the cytoplasmic domains of the two subunits are closely held together with a salt-bridge and the extracellular N-terminal head domains are in a bent conformation facing the plasma membrane (Askari et al., 2010; Kim et al., 2003). Both ligand binding and interactions between intracellular regulator proteins and the integrin tails can induce activation through conformational changes leading to the separation of the α- and β-subunits and extension of the head domain (Kim et al., 2003; Luo and Springer, 2006). The β-tail contains two conserved NPXY motifs (N, aspartic acid; P, proline; X, any amino acid; Y, tyrosine) which mainly function as recognition sites for proteins containing phosphotyrosine binding (PTB) domains. Binding of the protein 4.1, ezrin, radixin, moesin (FERM) domain of the cytoplasmic protein talin to the membrane-proximal NPXY motif of the β-subunit cytoplasmic tail is thought to be a key step in the activation of several integrins (Nieswandt et al., 2007; Simonson et al., 2006; Tadokoro et al., 2003). Talin is presumed to alter the tilt angle of the β-integrin transmembrane domain and disrupt interactions between the α- and β-subunits, and this ability makes it a unique activator

of integrins (Wegener et al., 2007). Recently, the importance of the β-integrin binding protein kindlin as a modulator of integrin activaton has also been appreciated. In contrast to talin, members of the kindlin family bind to the membrane-distal NPXY motif of the β-tail and they function as regulators of talin-dependent integrin activation (Harburger et al., 2009; Ma et al., 2008).

Unlike many other transmembrane receptors, integrins are unusual in their ability to mediate bidirectional signaling. Signals originated inside the cell or from other cell surface receptors can affect the affinity of the integrin for its extracellular ligand, thereby modifying cell-ECM interactions and transmitting forces required for migratory processes or ECM remodeling. Extracellular ligand binding is also able to trigger downstream signaling events inside the cells that result in different kind of responses. Integrins are able to induce phosphorylation-dependent signal transduction and local accumulation of the second messengers phosphatidylinositol 4,5 bisphosphate (PIP_2) and phosphatidylinositol 3,4,5 trisphosphate (PIP_3), cell polarization, spreading and migration and eventually gene expression changes resulting in for example survival cues. Integrin conformational changes are followed by lateral clustering in the membrane which is necessary for strong ECM interactions. Activated ligand-bound integrins form relatively unstable structures termed nascent adhesions that either disassemble or mature into focal complexes and subsequently larger focal adhesions (FAs). Focal adhesions are large, dynamic protein complexes that are assembled at the cytoplasmic side of clustered integrins and mediate the link between integrins and the actin cytoskeleton. The cytoplasmic tails of β-integrins are only 20-50 amino acids long and the integrins themselves do not possess any catalytic activity. Recruitment of various adaptor and scaffolding proteins that bind to specific sites in the integrin tails is therefore necessary for efficient organization of the cytoskeleton and induction of downstream signaling (Figure 3). In addition to binding and activating integrins through its head domain, talin also simultaneously binds to actin via its rod domain and thus functions a direct mechanical link between integrins and the actin cytoskeleton (Zhang et al., 2008b). Vinculin is recruited to nascent adhesions and is important in the growth and maturation of FAs. Vinculin binds directly to actin and the talin rod, thereby acting as a crosslinker and stabilizes actin-talin interactions (Humphries et al., 2007). Other essential adaptor proteins are paxillin, which modulates the composition of FAs by acting as a molecular platform that allows many simultaneous binding partners (Turner and Miller, 1994), and tensins, which crosslink integrin and actin (Lo et al., 1994). α-actinin and the major scaffold protein integrin-linked kinase (ILK) further strengthen integrin-cytoskeleton linkages (Laukaitis et al., 2001; Wang et al., 2008). Actin dynamics are regulated at focal adhesions, mainly through the Rho GTPases Rac, Cdc42 and RhoA (Legate et al., 2009). The physical link between integrins and actin further facilitates integrin-dependent regulation of cytoskeletal reorganization.

Focal adhesions are also sites of phosphorylation events, which serve to activate downstream targets and recruit a multitude of proteins. The integrin β-tail itself can be phosphorylated which is thought to act as a signaling switch that modulates the binding

pattern of different proteins. For example, phosphorylation of the membrane-proximal NPXY motif was shown to reduce talin binding while Dok1, which negatively regulated activation, bound more tightly (Anthis et al., 2009; Oxley et al., 2008). Tensin has been shown to be insensitive to integrin phosphorylation and is thought to replace talin at the β-tail and thereby induce adhesion strengthening and signaling (Legate and Fassler, 2009; McCleverty et al., 2007). β1-integrin phosphorylation has been studied by using single (β1Y783F or β1Y795F) or double (β1YY783,795FF) mutations of the NPXY motifs where the tyrosine residues have been substituted with nonphosphorylateable phenylalanines. Single substitutions have little effect on integrin conformation and signaling, while the double β1YYFF mutation increases the number of focal adhesions, alters cytoskeletal structure and decreases cell migration when adhesion is mediated via β1-integrin (Sakai et al., 1998). Phosphorylation of paxillin and tensin were also decreased in β1YYFF MEFs, as well as activation of FAK by Y397 and Y861 phosphorylation (Meves et al., 2011; Wennerberg et al., 2000). It has been shown that YYFF β1 tail peptides show ~50% decreased binding to talin and kindlin compared to wild-type peptides (Meves et al., 2011). β1YYFF mutant mice nevertheless show no obvious disease phenotype, while substituting the tyrosines of the NPXY motifs with alanines, which lack an aromatic ring structure, almost completely eliminated β1-integrin function *in vivo,* and talin and kindlin binding *in vitro* (Chen et al., 2006; Moser et al., 2008). The NPXY motifs are therefore essential for integrin activation, while tyrosine phosphorylation seems to be dispensable although it reduces activator binding (Chen et al., 2006; Czuchra et al., 2006; Meves et al., 2011). In a carcinogenesis model using TPA, β1YYFF mutant mice developed fewer tumors than wild-type mice. v-Src has been shown to phosphorylate β1 and this has been proposed to promote transformation (Sakai et al., 2001). However, simultaneous overexpression of Src kinase and TPA treatment led to similar tumor rates in β1YYFF and wild-type mice, suggesting that β1 phosphorylation does not play a role in Src-mediated transformation. Similar results were obtained with β1YYFF keratinocytes in a colony forming tumorigenesis assay. Also, Src expression rescued the reduced phosphorylation of FAK detected in β1YYFF keratinocytes (Meves et al., 2011).

In total, more than 180 molecules are proposed to be part of the integrin adhesome, including tyrosine kinases and phosphatases, actin-binding proteins, GEFs, GAPs and GTPases (Geiger and Zaidel-Bar, 2012; Zaidel-Bar and Geiger, 2010). In addition, integrin heterodimer composition and differences in tension dictate the exact components of the adhesome, emphasizing the complexity of FA adhesion structure, formation, turnover and signaling.

2.2.3.1 Src and Focal adhesion kinase

FAK is a ubiquitously expressed signaling scaffold that contains an N-terminal FERM-domain, a kinase domain, proline-rich regions and a C-terminal focal adhesion-targeting (FAT) domain (Figure 4). FAK interacts with the cytoplasmic tail of β1-integrin either directly through its FERM domain or via talin and paxillin that interact with the FAT-domain (Hayashi et al., 2002; Schaller et al., 1995). FAK is auto-

inhibited by interactions between the FERM-domain and the kinase domain that protects tyrosines Y576 and Y577 in the activation loop from Src-mediated phosphorylation (Lietha et al., 2007). Integrin ligation leads to displacement of the FERM-domain and auto-phosphorylation of FAK at tyrosine residue 397, which creates a binding site for the Src homology 2 (SH2) domain of Src (Schaller et al., 1994). Src also binds to the newly available proline-rich regions of FAK via its Src homology 3 (SH3) domain. In addition to integrins, other binding partners of the FAK-FERM domain are able to activate FAK, for example PIP_2 that locally accumulates at integrin ligation sites (Cai et al., 2008). FAK is also thought to be activated by integrin clustering which in turn promotes FAK clustering and *trans*-auto-phosphorylation, followed by release of the auto-inhibition via interactions with binding partners (Frame et al., 2010).

Src belongs to the Src family kinases (SFK) of non-receptor tyrosine kinases. In addition to SH2 and SH3 domains, Src also consists of a kinase domain, a regulatory C-terminal domain and an N-terminal myristoylation moiety important for its localization at the perinucelar, endosomal and plasma membrane compartments (Figure 4). Like other members of the SFK family, Src is regulated by intramolecular interactions and by a C-terminal tyrosine residue at Y527. When phosphorylated, this residue binds to the SH2 domain and auto-inhibits the kinase activity of Src. The tyrosine kinase Csk phosphorylates Src Y527 and integrin ligation leads to the dissociation of Csk from adhesion sites, leading to Y527 dephosphorylation and the subsequent accessibility of SH2 and SH3 binding partners, such as FAK (Figure 4). Direct dephosphorylation of the negative regulatory site by for example receptor tyrosine phosphatase alpha (RPTPalpha) or protein tyrosine phosphatase 1B (PTP1B) can also take place in response to integrin activation (Liang et al., 2005; Su et al., 1999). Upon FAK binding, Src is auto-phosphorylated at Y416 and phosphorylates and activates several downstream adaptors and kinases, among others paxillin, tensin, $p130^{CAS}$, p190RhoGAP and FAK (Arthur et al., 2000; Playford and Schaller, 2004; Schlaepfer and Hunter, 1996). $p130^{CAS}$ is a large docking protein that recruits adapters Nck and Crk to adhesion sites. Src phosphorylation at FAK Y925 creates a binding site for the adaptor protein Grb2 and the Ras GEF SOS (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996). The Src family protein kinases can also bind directly to β-integrins (Arias-Salgado et al., 2003) and phosphorylate the NPXY-motifs on the β-integrin tail.

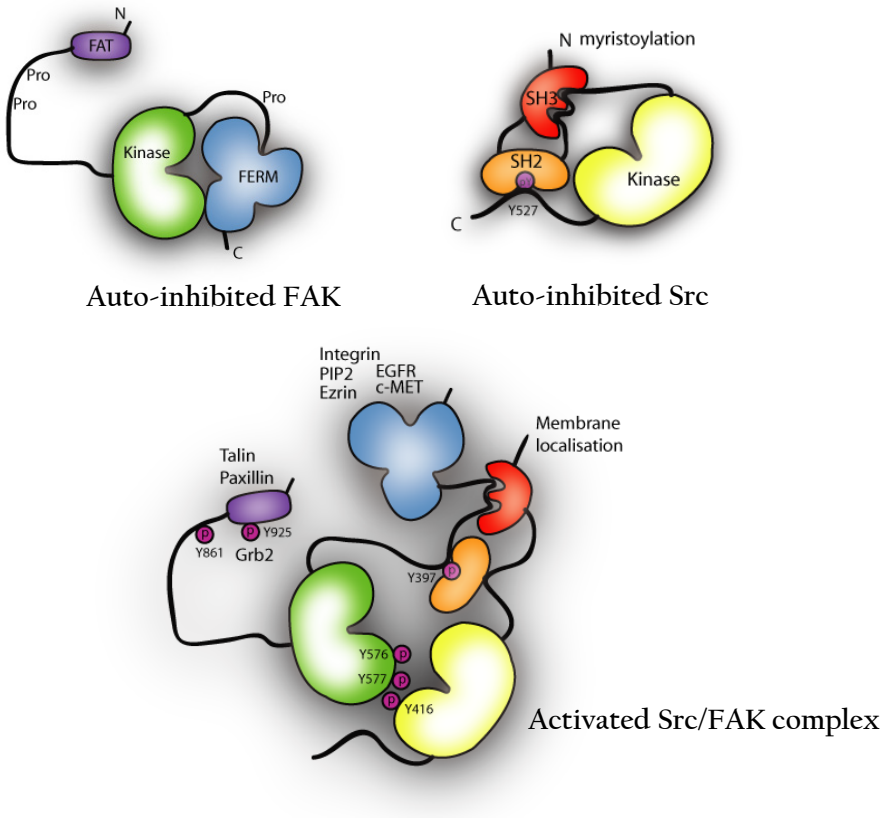

Figure 4. Structure and activation of Src and FAK. In their inactive conformations, FAK is auto-inhibited by interactions between the FERM-domain and the kinase domain, while Src is auto-inhibited by SH2 domain binding to the inactivating C-terminal phosphorylation site and interactions between the SH3 domain and the SH2-kinase linker region. Binding of the FAK-FERM domain to different binding partners leads to auto-phosphorylation of FAK at Y397 and the formation of a Src-FAK complex. Src, which in turn is auto-phosphorylated on Y416, phosphorylates FAK at several residues resulting in full activation. Some interaction partners for the specific domains are indicated. P, phosphate group; Pro, proline rich region. Adapted from Frame et al., 2010.

The wide range of Src substrates makes this kinase a potent activator of many pathways and it is usually found in its active state at focal adhesions or other peripheral structures. The kinase activity of Src has been shown to be dispensable for targeting of Src to the plasma membrane, while its SH3 domain is necessary for association with binding partners at the cell periphery. RhoA-mediated stress fibers are also required for delivery of Src from perinuclear compartments to the plasma membrane (Timpson et al., 2001). Using expression of Src-GFP fusion proteins in Src/Yes/Fyn$^{-/-}$ (SYF) MEFs Sandilands et al. showed that most of the Src pool remained inactive in the perinuclear region after stimulation of Rac, RhoA or Cdc42

(Sandilands et al., 2004). Active Src was located at the plasma membrane upon stimulation, but a small proportion was also detected inside the cell. Active Src was found to co-localize with the RhoGTPase RhoB in endosomes and in structures throughout the cytoplasm. These Src/RhoB containing structures also associated with actin filaments, and inhibition of actin polymerization or depletion of RhoB prevented accumulation of active Src at peripheral locations (Sandilands et al., 2004; Seong et al., 2009). In addition, impairing recycling with dominant-negative Rab11 also inhibited Src activation and translocation (Sandilands et al., 2004). This mechanism is thought to protect Src from becoming active at inappropriate sites in the cell. In contrast to other SFKs, Src is not palmitoylated and this is what distinguishes Src from the SFKs Fyn and Yes in terms of activation and membrane targeting to RhoB structures (Sandilands et al., 2007). Furthermore, disruption of the endosomal-sorting complexes required for transport (ESCRT) pathway leads to accumulation of active Src in late endosomes/ lysosomes and reduces localization at focal adhesions in MEFs and HeLa cells. This also impaired cell migration (Tu et al., 2010). The ESCRT pathway is needed for lysosomal degradation of proteins, but has also been shown to be important for recycling of molecules and targeting proteins to the plasma membrane. In this study, however, expression of dominant-negative Rab11 had no effect on the localization of active Src at FAs (Tu 2010). Integrins and Src have also been proposed to traffic together, as colocalization of pSrc, α5-integrin and the endosomal marker EEA1 has been observed in ESCRT depleted cells (Lobert and Stenmark, 2012).

2.2.4 Integrin traffic

Endocytosis of membrane-bound receptors is a way for cells to terminate or modulate signaling at the cell surface. Defects in many pathways that regulate the trafficking of growth factor receptors or cell adhesion molecules have in recent years been found to be a common characteristic of cancer cells. Failure to disassemble signaling complexes at the cell surface may lead to overexpression of growth signals, while aberrant trafficking of cell-cell junction proteins often lead to loss of epithelial cell polarity (Mosesson et al., 2008). Integrin traffic is needed for cell motility, cell division and focal adhesion turnover and derailed integrin traffic has been established as an important factor contributing to increased migratory and invasive properties of tumor cells.

2.2.4.1 Integrins internalize via different routes

To enter cells, integrins can use several internalization routes depending on the composition of the heterodimer, cell type and cellular context. Clathrin-dependent endocytosis is characterized by clustering of the cargo molecules within membrane structures coated with the triskelion protein clathrin (clathrin-coated pits), followed by membrane invagination and vesicle fission which is catalyzed by the GTPase dynamin. Adaptor proteins such as AP2, Numb and DAB2, recruit and bind cargo proteins in the coated pits (McMahon and Boucrot, 2011). After endocytosis the vesicles are uncoated and fuse with early endosomes. Clathrin-dependent endocytosis of integrins occurs through different mechanisms. For example, HS1-associated

protein X1 (HAX-1) promotes migration and invasion of carcinoma cells by binding αvβ6 and regulating its endocytosis via the clathrin-dependent route (Ramsay et al., 2007). The adaptor protein DAB2 was found to colocalize with β1-integrin in coated pits and regulate its endocytosis. Increased surface levels of β1, α1 and α2 subunits (but not αv or α5) were detected in DAB2-deficient HeLa cells (Teckchandani et al., 2009; Teckchandani et al., 2012). DAB2, AP2 and dynamin are also needed for efficient focal adhesion disassembly, which was shown to require clathrin-dependent endocytosis of active β1-integrin (Chao and Kunz, 2009; Ezratty et al., 2009). Some clathrin adaptors, including DAB2 and Numb, contain PTB domains that are able to bind NPXY motifs. NPXY motifs function as sorting signals in several transmembrane proteins (Bonifacino and Traub, 2003) and these motifs have also been implicated in internalization of integrins. The adaptor protein Numb can bind directly to β1 and β3 integrins (Calderwood et al., 2003) and knockdown of Numb inhibits endocytosis of both these integrins as well as migration on specific substrates (Nishimura and Kaibuchi, 2007). In addition, expressing a tyrosine to phenylalanine substitution in both NPXY motifs of β1-integrin (β1YY783,795FF) in β1 null GD25 mouse embryonic fibroblasts (MEFs) causes reduced clathrin-mediated endocytosis. In β1YYFF MEF cells isolated and cloned from E13.5 embryos which carry the mutation in germline (Czuchra et al., 2006), integrin endocytosis is also significantly reduced (Pellinen et al., 2008). Interestingly, overexpression of the small GTPase Rab21, which has been shown to bind the α-tail and induce integrin internalization and recycling, rescues the decreased endocytosis of β1YYFF cells (Pellinen et al., 2006; Pellinen et al., 2008). This could be due to induction of an alternative, clathrin-independent endocytosis route.

Integrins also employ clathrin-independent routes to enter cells, often via caveolin-1-dependent caveolae. Caveolae are invaginations in the plasma membrane that typically contain lipid raft molecules like sphingolipids, cholesterol and caveolin (Le Roy and Wrana, 2005). The serine/threonine kinase protein kinase Cα (PKCα) resides in the caveolar membrane and its activity regulates internalization of caveolae (Smart et al., 1995). PKCα can bind directly to the integrin β-tail and it promotes integrin internalization and thereby cell motility (Ng et al., 1999). Also, caveolin-1 colocalizes with αvβ3 and MT1-MMP (Galvez et al., 2004) and with the fibronectin receptor α5β1 in endothelial cells (Wickstrom et al., 2002). α5β1 endocytosis has been shown to be dependent on caveolin-1 and fibronectin matrix turnover is controlled by receptor-mediated endocytosis. Knockdown of caveolin-1 consequently affects not only α5β1 internalization but seemingly also ECM remodeling (Shi and Sottile, 2008). Interestingly, ECM stiffness also determines endocytosis rates since bone marrow mesenchymal stem cells showed enhanced caveolae-dependent internalisation on soft substrates (Du et al., 2011). The importance of cholesterol-rich caveolae is also underlined by the observation that αLβ2 and α4β1 localize to lipid rafts in the plasma membrane (Leitinger and Hogg, 2002). Cholesterol depletion inhibits cell migration and αLβ2 endocytosis in leukocytes (Fabbri et al., 2005) and β1-integrin endocytosis in intestinal epithelial cells (Vassilieva et al., 2008). Caveolar endocytosis also requires the catalytic activity of dynamin, but integrins are

also thought to internalize via clathrin- and dynamin-independent pathways (Howes et al., 2010) and via circular dorsal ruffles in macropinocytosis (Gu et al., 2011).

2.2.4.2 Trafficking determines function

Regardless of their mode of entry, integrins mostly end up in the early endosome after internalization. Early endosomes are key hubs for controlling the fate of endocytosed receptors; from here they are usually recycled back to the plasma membrane or sent to degradation to the lysosome. Members of the Rab and Arf family of small GTPases are generally important for intracellular trafficking as they are involved in for example selection of cargo molecules, recruitment of effector proteins, regulating the lipid composition in membranes and vesicle fusion and transport (D'Souza-Schorey and Chavrier, 2006; Hutagalung and Novick, 2011). After internalization, most integrins find their way back to the cell surface, either directly from the early endosome via a Rab4-dependent pathway, or through the perinuclear recycling compartment from where they can take a Rab11-and Arf6- dependent recycling route (di Blasio et al., 2010; Powelka et al., 2004; Roberts et al., 2001; Yoon et al., 2005). The GTPase activating protein p120 RasGAP has also been shown to replace Rab21 on the integrin α-tail in early endosomes and thereby promote recycling of the integrin heterodimer (Mai et al., 2011). Different trafficking routes largely determine migratory and invasive capacities of cells and also function to restrict signaling to specific subcellular localizations, explaining the alterations in recycling pathways detected in malignant cells. Gain-of function mutations in p53 are frequently detected in cancer and mutant p53 has been shown to bind and inactivate p63, thus promoting tissue invasion through increased recycling of α5β1 and EGFR (Muller et al., 2009). This requires the interaction between α5β1 and Rab-coupling protein (RCP) and formation of an RCP-integrin-EGFR complex. Blocking ligand binding of αvβ3 also induces this α5β1 recycling pathway and promotes rapid migration on 2D surfaces and invasion into 3D matrices (Caswell et al., 2008). In ovarian cancer cells invasion in 3D matrixes (but not 2D) is driven by the association between integrin β1 and the small GTPase Rab25. This interaction promotes localized recycling of β1-integrins to the tips of cell protrusions that extend into the matrix at the cell front, while also inducing retraction of the cell body through controlling integrin recycling in the cell rear (Caswell et al., 2007; Dozynkiewicz et al., 2012).

In addition to the well-established role in migratory processes, regulation of integrin traffic is also critical during cell division. In mid-telophase during mitosis integrins are localized to the basal side of the cleavage furrow where they seemingly function to anchor the structure to the underlying substrate. In late telophase, protrusions are formed at the opposite poles of the two daughter cells. Integrins are found in these structures, where they provide mechanical support for the cells to separate (Pellinen et al., 2008). Inhibition of Rab21 binding to the integrin α-tail, Rab21 activity or integrin traffic by other means results in delayed or failed cytokinesis and thus formation of bi-and multinucleate cells. β1YYFF MEF which have defective endocytosis, also show impaired cytokinesis on β1-specific substrates. In addition, ovarian carcinoma cells harboring a deletion in the *RAB21* locus become increasingly

multinucleate in cell culture. Integrin localization at the cleavage furrow also correlates with RhoA activity, suggesting that integrins also mediate important signaling cues necessary for cytokinesis, in addition to providing anchorage (Pellinen et al., 2008). Rab21 activity and functional integrin traffic is thus needed to execute cytokinesis and deregulated integrin traffic could consequently promote genomic instability and tumorigenesis through cell division defects. Deregulated integrin traffic in cancer is also highlighted by the observation that hypoxia can initiate Rab11-recycling of α6β4 in MDA-MB-231 breast carcinoma cells (Yoon et al., 2005) and several Rab GTPases have been found to be deregulated in different cancers (Cheng et al., 2004; He et al., 2002).

2.3 Syndecans

The syndecans are a family of cell surface heparan sulfate proteoglycans. There are four members of this family, syndecan-1-4, and each member consists of an extracellular ectodomain, a conserved transmembrane domain and an intracellular domain (Figure 5). The ectodomain is covalently linked to heparan sulfate (HS) or chondroitin sulfate (CS) GAG chains that function as the ligand binding sites of syndecans. Syndecans form functional homodimers through self-association of the transmembrane domain. They also bind to growth factors and ECM proteins, although the syndecan binding sites on matrix proteins are distinct from those of integrins. The cytoplasmic domains of syndecans contain two conserved regions, C1 and C2, as well as a variable region that is unique to each syndecan. The C1 region interacts with different cytoskeletal proteins, while C2 binds to proteins containing PDZ (PSD95 (postsynaptic density protein of 95 kDa), Discs large, Zonula occcludens 1) domains (Tkachenko et al., 2005). The V region has been shown to be important for cell migration and matrix assembly (Chakravarti et al., 2005; Klass et al., 2000) and this region binds to for example PIP_2 and PKCα in syndecan-4 (Horowitz et al., 1999; Oh et al., 1997). The expression of syndecan-1-3 are largely tissue-specific while syndecan-4 is more widely distributed. Syndecan-1 is needed in developmental processes and is also found in mature epithelial cells, syndecan-2 is mostly expressed in fibroblasts, endothelial cells and neurons and syndecan-3 is mainly important in development (Ethell and Yamaguchi, 1999; Fears et al., 2006; Hinkes et al., 1993; Kosher, 1998). Syndecan-4, on the other hand, is expressed in both developing and most adult tissues.

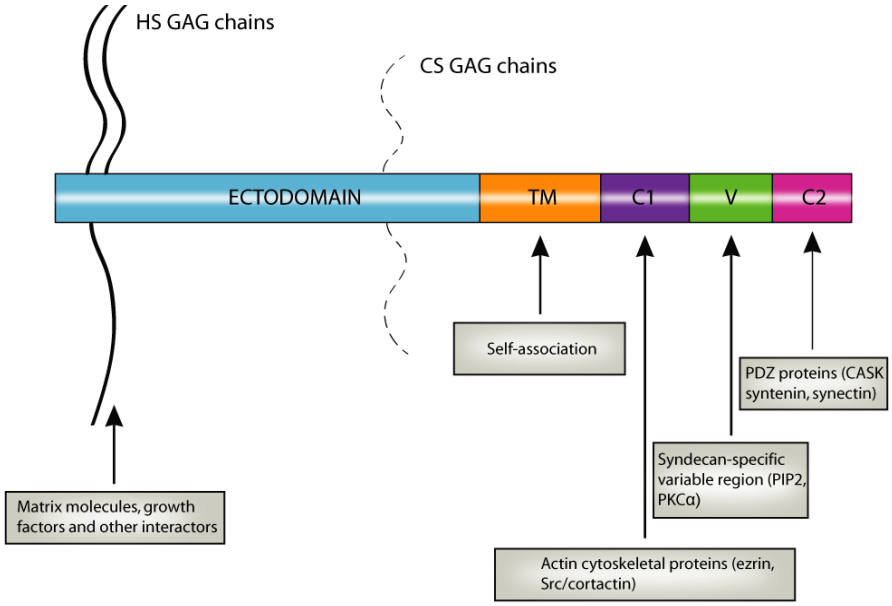

Figure 5. Syndecan structure. Functions and interactions of the specific domains on the syndecan molecule are indicated. ECTO, ectodomain; TM, transmembrane domain; C1 and C2, conserved regions; V, variable region. See text for details. Adapted from Couchman, 2003.

2.3.1 Syndecan signaling and cooperation with integrins

Syndecans often function as co-receptors for other cell surface receptors, but are also able to transmit signals on their own even though they lack intrinsic kinase activity. In most cell types, both integrin and syndecans are needed for a full response to cell adhesion. Using overexpression and knockdown of different syndecans, it was shown that syndecan-1, but not syndecan-2 or -4, supports α2β1 mediated adhesion to collagen in chinese hamster ovary (CHO) cells and breast carcinoma MDA-MB-231 cells. This co-operation is necessary on both monomeric and fibrillar collagen, but not on adhesion to the integrin–specific GFOGER peptide, and requires the presence of HS-GAG chains. Syndecan-1 also regulates organization of actin fibres and collagen-mediated induction of MMP-1 (Vuoriluoto et al., 2008). Syndecan-1 is also a co-receptor for α2β1 in head and neck cancer, given that cell adhesion is reduced on collagen I in response to syndecan-1 silencing in these cells (Ishikawa and Kramer, 2010). In addition, syndecan-1 cooperates with αvβ3 in mammary carcinoma cells and with αvβ5 in mouse fibroblast cells spreading on vitronectin. In both cases this requires the ectodomain of syndecan-1 (Beauvais et al., 2004; McQuade et al., 2006) and the inhibitor synstatin, a peptide based on the syndecan ectodomain, disrupts the integrin-syndecan interaction and decreases angiogenesis *in vitro* and *in vivo* (Beauvais et al., 2009). Activation of αvβ3 by syndecan-1 involves the recruitment and activation IGF1R, which subsequently activates the integrin via an inside –out talin-dependent mechanism (Beauvais and Rapraeger, 2010). On laminin, syndecan-1 modulates integrin α6β4 signaling (Ogawa et al., 2007) and cooperation between syndecans and α2β1 has also been observed (Hozumi et al., 2006). Syndecan-4 is the only family

member present at focal adhesions and its synergistic signaling with α5β1 on fibronectin is well established (Woods et al., 2000). Syndecan-4$^{-/-}$ fibroblasts adhering to fibronectin or normal rat fibroblasts adhering to fibronectin lacking syndecan binding sites also show reduced activation of FAK and less tyrosine phosphorylation overall (Kim et al., 2001; Wilcox-Adelman et al., 2002). Although syndecan-4 is the only member found at FAs, syndecan-2 has also been shown to bind the heparin-binding domain of fibronectin (HepII) and to promote FA formation in some cell types (Kusano et al., 2000; Munesue et al., 2002). Syndecan-2 signaling may thus be needed for organizing membrane structures preceding FA assembly. Also, in a pathway exclusive to mesenchymal cells, the ectodomains of syndecan-2 and-4 alone were able to induce integrin-dependent spreading and signaling (Whiteford et al., 2007).

Syndecans can via their long and flexible GAG chains catch and bind growth factors and other ligands that are far away from the core of the receptor. Depletion of GAG chains disrupts binding of FGF and VEGF leading to reduced growth factor receptor-mediated signaling (Fuster et al., 2007). Both FGF and FGFR bind directly to HS or the closely related heparin, the presence of which are required for a full signaling response downstream of FGFR (Harmer, 2006). The highly conserved C2 region of syndecans binds PDZ domain proteins, including syntenin, synectin, synbindin and CASK (Ca/calmodulin associated serine/threonine kinase). PDZ proteins often function as scaffolds and may thus promote syndecan oligomerization and recruitment of additional signaling molecules. As an example, syntenin can also bind PIP_2 and the PIP_2-syntenin complex controls syndecan recycling together with Arf6. Disruption of syntenin- PIP_2 binding leads to intracellular accumulation of syndecans and reduced cell spreading (Zimmermann et al., 2005). The C1 domain of syndecan-2 has been shown to bind the cytoskeletal protein ezrin, which serves as a crosslinker between actin and the syndecan (Granes et al., 2000; Granes et al., 2003). Syndecan-3 C1 domain interacts with a complex of Src and cortactin and clustering of syndecan-3 is able to activate Src signaling (Kinnunen et al., 1998). Concerning other binding partners of the cytoplasmic region, most details have been acquired for syndecan-4. Syndecan-4 is unique in its ability to recruit PKCα to focal adhesions and activate the kinase. Indeed, syndecan-4 mediated activation of PKCα is required for FA formation on fibronectin. Cells plated on an integrin-specific ligand that lacks binding sites for syndecan-4 consequently fail to form focal adhesions (Woods et al., 2000). In response to for example FGF2, PKCα binds to the V region of syndecan-4 and is activated following formation of a complex containing syndecan-4, PKCα and PIP_2 (Lim et al., 2003; Oh et al., 1998). PIP_2 is able to bind directly to the V region of syndecan-4, but also interacts with the regulatory domain of PKCα (Corbalan-Garcia et al., 2003; Horowitz et al., 1999). Signaling is terminated by PKCδ phosphorylation of syndecan-4 at S183, which also disrupts syndecan oligomerization (Koo et al., 2006; Murakami et al., 2002). Phosphorylation prevents the interaction with syntenin, but favors syndecan-4 binding to the actin-bundling protein α-actinin, providing a link to the cytoskeleton (Chaudhuri et al., 2005; Greene et al., 2003). Syndecan-4 is involved in regulating endocytic pathways, considering its interaction with dynamin 2 and that PKCα controls β1 internalization via direct binding to its cytoplasmic tail (Ng et al.,

1999). Indeed, syndecan-4 regulates the surface availability of integrins during wound healing by PKCα mediated activation of RhoG, a small GTPase involved in internalization of growth factor receptors. Syndecan-4 and PKCα releases the inhibitor RhoGDI1 from integrin β1, which causes activation of RhoG and subsequent α5β1 integrin endocytosis (Bass et al., 2011; Elfenbein et al., 2009), a process that promotes adhesion site turnover. This turnover is needed in wound healing, demonstrated by the impaired wound closure detected in $RhoG^{-/-}$ mice (Bass et al., 2011).

Syndecan-4 signaling in particular is able to activate the Rho family of small GTPases, leading to cytoskeletal and morphological changes. Syndecan-4-mediated PKCα activation results in localized activation of Rac1 at the leading edge of fibroblasts, resulting in increased directional migration (Bass et al., 2007). In these cells, syndecan-4-fibronectin engagement simultaneously suppressed RhoA and promoted Rac1 activity. In the absence of ligand, however, syndecan-4 suppressed Rac1 activity (Bass et al., 2007). Syndecan-4 has also been shown to internalize together with FGF2, and this process is dependent on syndecan-4 activation of Rac1, as well as Cdc42 regulated macropinocytosis (Tkachenko et al., 2004). PKCα activation also increases the formation of stress fibers and GTP-loaded RhoA in rat fibroblasts (Dovas et al., 2006). RhoA activity was reduced in response to RNAi-mediated silencing of PKCα or the use of dominant negative PKCα. However, direct RhoA activation by LPA via a G protein-coupled receptor pathway bypasses the need for syndecan-4 or PKCα (Dovas et al., 2006). Integrin α5β1 is known to regulate RhoA via inducing Src- and Arg-dependent phosphorylation and activation of p190RhoGAP (Arthur et al., 2000; Bradley et al., 2006). Syndecan-4 is a co-regulator of RhoA suppression as PKCα activation is necessary for the proper intracellular localization of p190RhoGAP (Bass et al., 2008). Syndecan-4 is thus thought to promote migration by activating Rac1 as a first response to induce membrane protrusions, while cell contraction and maturation of adhesions would happen later through activation of RhoA (Brooks et al., 2012). In head and neck squamous cell carcinoma, Syndecan-1 depletion reduces actin polymerization, the formation of focal adhesions and FAK and RhoA activation, while Rac1 activity was increased, as well as migration and invasion on collagen I matrix. Inhibition of the RhoA effector Rho kinase (ROCK) resulted in the same phenotype, suggesting that syndecan-1 reduces cell motility through the activation of RhoA in this cell type (Ishikawa and Kramer, 2010).

3. ANEUPLOIDY

Most eukaryotic organisms contain a diploid set of chromosomes, namely one haploid set of chromosomes derived from each parent. Some plants and fungi also spend most of their life cycle in a haploid state, but polyploidy, the acquisition of one or more additional sets of chromosomes, is even more widespread in nature (Otto and Whitton, 2000). Polyploidy is very common in plants and can also be the result of normal development, for example in the salivary glands of *Drosophila melanogaster* or mammalian hepatocytes and megakaryocytes (Storchova and Pellman, 2004).

Although whole-organism polyploidy is very rare in mammals, polyploidization of the genome, or segments of it, is thought to have been important in the evolution of the vertebrate lineage (Makalowski, 2001). Aneuploidy, in contrast, is a deviation from a normal haploid, diploid or polyploid chromosome complement and is not an exact multiple of the haploid chromosome number. It often results in apoptosis and is associated with disease and sterility (Torres et al., 2008). In addition to whole-chromosome aneuploidy, structural aberrations such as deletions, translocations or inversions also occur. These chromosomal abnormalities can be the result of defects in various components of the cell division machinery.

3.1 Causes of aneuploidy

Aneuploidy can arise directly in diploid cells through amplification of centrosomes, inappropriate attachment of chromosomes to the mitotic spindle and deregulation of mitotic checkpoint proteins (Kops et al., 2005). The centrosome is the main microtubule-organizing centre which nucleates microtubule growth and forms the poles of the bipolar spindle in mitosis. Centrosomes are important for the accuracy of chromosome segregation and are required for completion of cytokinesis (Piel et al., 2001). Supernumerary centrosomes could be a consequence of abnormal centrosome replication and this can lead to a multipolar mitosis which ultimately causes whole-chromosome aneuploidy.

Mitosis is under regulation of several protein complexes and the mitotic checkpoint, or spindle assembly checkpoint (SAC), is an important control system for proper segregation of chromosomes. The SAC prevents missegregation of chromosomes by delaying advance to anaphase until the kinetochores of all chromosomes have attached to the spindle microtubules. It appears to monitor both kinetochore-microtubule attachment and tension between the sister chromatids generated from this attachment (Musacchio and Salmon, 2007). The SAC contains about ten distinct proteins interacting to prevent premature anaphase and thus functions to prevent aneuploidy. Some mitotic defects do not activate the SAC, such as merotelic attachments that occur when microtubules from both spindle poles attach to the same kinetochore. Merotely often leads to lagging chromosomes and missegregation (Weaver and Cleveland, 2006). Aneuploidy could also be the result of either prolonged cohesion or premature loss of cohesion between the sister chromatids.

Chromosome segregation errors have recently been shown to not only induce whole-chromosome aneuploidy, but also structural aberrations (Janssen et al., 2011). Inducing segregation errors in human retinal pigment epithelial cells (RPE-1) by inhibition of the SAC protein Mps1 or treatment with the merotely-inducing drug Monastrol, resulted in abnormal nuclei with damaged DNA and chromosomal translocations. DNA damage was also detected in the CIN cancer cell lines MCF7 and SW480, which show spontaneous segregation errors. It is thought that forces generated by the cleavage furrow are responsible for breaking missegregating chromosomes, because blocking cytokinesis in these cells decreased the appearance of markers for double-

strand breaks. As in other cell types, chromosome missegregation promoted a p53-dependent arrest in G1.

Aneuploidy is also thought to arise through a polyploid intermediate. Polyploid cells can be the result of among other things cell fusion, endoreplication or an abortive cell cycle due to mitotic slippage or failure of cytokinesis (Ganem et al., 2007). Cell fusion is a programmed step in the normal development of some cell types, but it can also occur during viral infections or spontaneously in cell culture (Ogle et al., 2005). Endoreplication also takes place during development and is a regulated way to generate polyploid cells by leaving out mitosis after DNA replication. This process is common in arthropods and plants and in mammals particularly in megakaryocytes, bone marrow cells that generate platelets (Edgar and Orr-Weaver, 2001). An abortive cell cycle on the other hand, generates polyploid cells due to defects in functions such as DNA replication, dissolving sister-chromatid cohesion, the mitotic spindle and cytokinesis. This occurs, in contrast to endoreplication, in pathological situations (Storchova and Pellman, 2004). In a process called mitotic slippage, cell cycle progression through mitosis is at first blocked through checkpoint responses. This arrest is, however, often transient and the cells thus become tetraploid by entering into the G1 phase of the cell cycle without completing mitosis or cytokinesis (Storchova and Kuffer, 2008). Cell adhesion receptors are important for cell cycle progression of normal cells, and matrix detachment of many anchorage-dependent cell types can result in cytokinesis failure and binucleation (Aszodi et al., 2003; Kanada et al., 2005; Reverte et al., 2006; Thullberg et al., 2007). The extracellular matrix of cartilage mainly consists of aggrecan and a collagen network and is produced by chondrocytes that surround themselves with ECM molecules. Chondrocytes have high levels of β1 and different α integrins and chondrocyte-specific β1 deletion in mice results in several defects, including formation of binucleate cells (Aszodi et al., 2003). Also, inhibiting integrin activity suppresses microtubule growth from the centrosomes and formation of the bipolar spindle, resulting in delayed or failed cytokinesis (Reverte et al., 2006). Cells also fail cytokinesis when integrin traffic is inhibited (Pellinen et al., 2008). In addition, loss of cell adhesion can lead to entosis, where one cell is internalized into another cell leading to the formation of cell-in-cell structures. The internalized cell can be positioned so that it disrupts the cleavage furrow of the outer cell and thereby execution of cytokinesis, resulting in binucleation (Krajcovic et al., 2011).

Following induction of cytokinesis failure in culture, tetraploid cells often undergo a p53-dependent cell cycle arrest in the next G1 phase (Stukenberg, 2004) (Figure 6), which has led to a debate over the existence of a “tetraploidy checkpoint” that responds to some aspects involved in polyploidization (Andreassen et al., 2001). However, deviant centrosome numbers, cytokinesis failure induced by very low doses of the actin inhibitor cytochalasin or polyploidy as a result of cell fusion do not induce G1 arrest in cells containing intact p53 (Uetake and Sluder, 2004; Wong and Stearns, 2005). This p53-dependent cell cycle arrest may therefore be due to DNA damage and other cellular stresses associated with an abortive cell cycle. In response to DNA damage cells arrest in G1 and high concentrations of the CKI $p21^{Cip1}$, a downstream target of

p53, and activated Rb proteins can be detected. Tetraploid mouse embryonic fibroblasts (MEFs) lacking Rb or p53 fail to arrest in G1 (Borel et al., 2002). A duplicated number of centrosomes usually appear in tetraploid cells that have undergone an abortive cell cycle. Normal proliferation as tetraploids is possible if these centrosomes cluster into two poles and thereby enable a bipolar mitosis (Figure 6) (Borel et al., 2002). However, even though a bipolar spindle is formed, the presence of extra centrosomes has been shown to lead to a higher rate of merotely and missegregation in various cancer cell lines (Ganem et al., 2009; Silkworth et al., 2009). Cytokinesis failure could also lead to a chaotic, multipolar mitosis and aneuploidy (Figure 6).

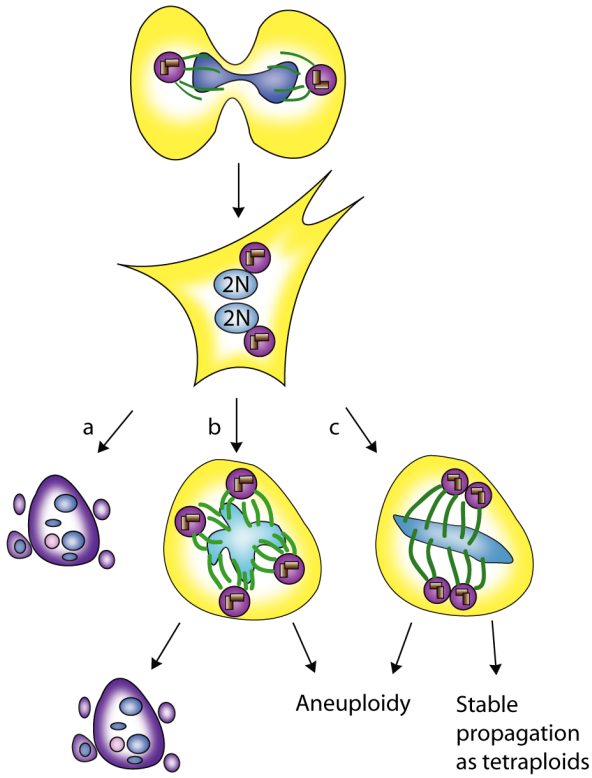

Figure 6. **Aneuploidy can arise form polyploid cells**. Cytokinesis failure leads to the formation of a binucleate cell. (a) In the presence of p53, the cells will arrest after the first tetraploid mitosis and eventually undergo cell death. (b) In p53-deficient cells, the polyploid cell can also produce aneuploid progeny via multipolar mitosis under certain conditions. Most cells probably die due to non-viable chromosome combinations, but rare aneuploid cells with proliferative capacity can also emerge. (c) Centrosome clustering can allow for stable propagation of tetraploid cells, but has also been shown to increase formation of lagging chromosomes, leading to chromosomal instability and aneuploidy. Adapted from Storchova and Pellman, 2004.

3.2 Consequences of aneuploidy

Aneuploidy is a common feature of many tumor types. However, whether it is just a consequence of malignant transformation or the actual cause of tumorigenesis has been a long-standing question. The idea that cancer could be the result of a deviant chromosome number was first proposed by German scientist Theodor Boveri nearly 100 years ago (Boveri, 2008). Based on observations in sea urchin eggs, Boveri suggested that multiple centrosomes might be the principal cause of cancer. Even though aneuploidy is highly associated with cancer, it also leads to developmental abnormalities in most species and in humans aneuploidy is often the reason behind mental retardation and miscarriages (Hassold and Hunt, 2001). The most common disease in humans due to aneuploidy is Down's syndrome which is a trisomy with an extra copy of chromosome 21 in all cells and the only autosomal trisomy that is viable in humans (Antonarakis et al., 2004). Other trisomies in humans include Edwards' syndrome (trisomy 18) and Patau's syndrome (trisomy 13), but these conditions have a very low rate of survival. The only viable human monosomy is Turner's syndrome in females with only one copy of the X chromosome. All other monosomies are embryonic lethal (Hassold and Hunt, 2001). Structural abnormalities or partial aneuploidy where differences in chromosome number are only manifested in some cells in the body are more tolerated (Emanuel and Shaikh, 2001).

3.2.1 Reduced fitness

Both chromosome-specific and common responses to aneuploidy have been detected by studying the transcriptional and proteasomal consequences of inducing aneuploidy in different cell models. On the organismal and cellular level, aneuploidy often causes a proliferative disadvantage, especially gains of extra chromosome copies (Pavelka et al., 2010). Using MEFs, Williams and co-workers determined the effects of an extra copy of chromosome 1, 13, 16 or 19, which all contain a relatively large number of genes (Williams et al., 2008). These trisomic MEFs proliferate more slowly and have an increased cell volume, increased glutamine consumption and increased ammonium and lactate production, reflecting an alteration in energy metabolism. The proliferation defect in aneuploid MEFs was also detected in yeast strains with extra chromosomes (Torres et al., 2007). In yeast, gain of extra chromosomes causes imbalances in the intracellular protein composition (Torres et al., 2007) which could be the reason behind the phenotypes seen in trisomic MEFs as well (Williams et al., 2008). Chromosomes 13, 18 and 21 in humans have the smallest number of transcripts and are the only trisomies tolerated after birth. This could be due to the relatively small amount of protein imbalances caused by these particular trisomies (Torres et al., 2008).

Interestingly, disomic yeast cells evolved to have improved proliferation rates contain specific mutations that allow them to tolerate the adverse effects of aneuploidy (Torres et al., 2010). 4 different disomic strains had different inactivating mutations in the deubiquitinating enzyme Ubp6, which leads to accelerated proteasomal degradation of specific substrates. Loss of Ubp6 is thought to improve growth by degrading excess proteins originating from the extra chromosome. In addition, these evolved strains displayed strain-specific gene expression changes, but also shared a common gene

expression pattern compared to the parental disomic (slowly proliferating) strains. Common responses included upregulation of genes involved in amino acid metabolism and downregulation of ribosomal and transport proteins (Torres et al., 2010).

Yeast cells have also been studied to determine whether the presence of extra chromosomes could lead to genomic instability (Sheltzer et al., 2011). In disomic haploid strains of *Saccharomyces cerevisiae* that carry one additional chromosome, the rate of chromosome missegregation and accumulation of Rad52-containg foci, a marker for recombinational repair, was increased. The presence of structural damage in the chromosomes is consistent with induction of segregation errors in mammalian cells (Crasta et al., 2012; Janssen et al., 2011). The disomic strains were also more sensitive to DNA-damaging drugs that induce double stranded-breaks (that are repaired by homologous recombination) and are likely defective in DNA repair mechanisms. The aneuploidy-induced CIN and increased mutagenesis are thought to be due to imbalances in different gene products or proteotoxic stress caused by superfluous proteins (Sheltzer et al., 2011).

Reduced growth rate was also reflected in the transcriptional profiles of disomic aneuploid yeast *Saccharomyces cerevisiae,* trisomic *Arabidopsis thaliana* plants, trisomic MEFs and trisomic human cells. Many of the same pathways were affected in yeast and plants in response to aneuploidy, as determined by gene ontology (GO) term enrichment. In the MEFs, an upregulated gene in one trisomic cell line was also likely to be upregulated in another trisomic cell line, though these MEFs harbored different extra chromosomes. The deregulated genes in the human trisomies also correlated well with that of the MEFs, with upregulation of genes related to the extracellular region, immune response and inflammatory response, and downregulation of genes related to the cell cycle and cell division. 254 ortholog genes were differentially expressed in the same direction in all species; 240 of them downregulated. Among the GO terms affected for the 240 genes were ribosome biogenesis, cell cycle and nucleic acid metabolism. Exposing the cells to different types of exogenous stresses revealed that the similarities in transcription among aneuploid cells are probably due to a conserved response to slow growth or stress, and not aneuploidy per se (Sheltzer et al., 2012). Similar results were obtained by analyzing DNA, RNA and protein levels of trisomic or tetrasomic human RPE-1 and HCT116 cells. Regardless of the identity of the extra chromosome, the aneuploid cell lines displayed an upregulation of pathways involved in Golgi and lysosome functions, lipid biogenesis and energy metabolism, while DNA replication and repair and mRNA processing were downregulated (Stingele et al., 2012). These also showed increased activity in autophagy, determined by the elevated levels of LC3-11 and p62 detected in the aneuploid cells. Autophagy is usually induced in response to stress or to produce energy when nutrients are low and is a process in which unnecessary or damaged organelles and proteins are degraded. Consistent with previous reports, all aneuploid cells proliferated poorly compared to isogenic diploid cells (Stingele et al., 2012).

These studies thus propose that numerical aneuploidy in yeast and mammalian cells induces a proteotoxic stress response, as aneuploidy in many cases increases the cells'

energy needs as well as proteasome function and protein folding (Sheltzer et al., 2012; Torres et al., 2007; Torres et al., 2010; Williams et al., 2008). Dosage compensation, the up-or downregulation of proteins to maintain correct stoichiometry, most likely takes place at least in yeast cells with subunits that are part of protein complexes (Stingele et al., 2012; Torres et al., 2010). Global responses to aneuploidy seem to be irrespective of the individual chromosomes and the genes that they contain as protein quality control pathways were upregulated in very different models of aneuploidy. Trisomic MEFs are consequently more sensitive to drugs that exaggerate the adverse effects of aneuploidy (i.e. increase proteotoxic stress) or impair pathways on which they are dependent for their survival. In these cells, the AMPK agonist AICAR induces already elevated levels of autophagy and Hsp72 and the Hsp90 inhibitor 17-AAG impairs proteasomal degradation. This leads to increased cell death compared to wild-type MEFs (Tang et al., 2011). Similar sensitivity towards these compounds was also detected in aneuploid human cancer cells. Compounds that exaggerate stress associated with aneuploidy could therefore potentially be used to specifically target aneuploid cancer cells (Tang et al., 2011).

3.2.2 Adaptation and phenotypic variability

Despite the observations that aneuploidy in many systems leads to a stress response, analysis of 38 stable aneuploid yeast strains of 35 different karyotypes did not reveal a global enrichment of genes related to stress among the differentially expressed genes compared to euploid strains (Pavelka et al., 2010). In this study, strains with similar karyotypes tended to have similar proteomic changes and displayed minimal dosage compensation. Interestingly, some of the aneuploid yeast strains showed increased fitness and improved growth under conditions that were suboptimal for euploid strains, for example in the presence of drugs or at lower temperatures (Pavelka et al., 2010). Both chromosome composition and growth conditions seem to determine the effect of aneuploidy on cell fitness in yeast strains. Thus, in contrast to the reduced fitness seen in many cell types under normal conditions, induction of aneuploidy may lead to evolution of adaptive phenotypes under stress conditions, thereby conferring a growth advantage (Pavelka et al., 2010). Although these strains were selected based on their karyotypic stability, later comparative genomic hybridization (CGH) analysis revealed that almost half of the strains displayed a heterogenous karyotype within the population (Sheltzer et al., 2011).

Another study showed that different stress conditions, including oxidative and translational stress, and especially inhibition of Hsp90, induce chromosome loss and gain in haploid yeast (Chen et al., 2012). Exposure to low doses of Hsp90 inhibitors also led to evolution of an aneuploid cell population that was more resistant to other forms of stress. Hsp90 has diverse roles in the cell, including in cell division and fidelity of chromosome segregation (de Carcer, 2004; McClellan et al., 2007). It is thought that cells allow survival of the population under environmental stress conditions by inducing aneuploidy, potentially through Hsp90 inhibition, and thereby promoting phenotypic variation (Chen et al., 2012).

Cancer evolution from benign to invasive correlates with increased aneuploidy and there is speculation over whether a particular combination of chromosomes, and therefore a particular gene expression profile, is important for the survival and proliferation of aneuploid cells. In carcinomas, chromosomal imbalances often comprise whole chromosomes or chromosome arms and they are thought to occur early in the neoplastic process (Ried et al., 2012). As much as 25% of the genome of a typical cancer sample contains arm-level copy number alterations (Beroukhim et al., 2010). Comparing CGH data and DNA ploidy from different studies shows a pattern of non-random genetic aberrations specific for different types of tumors (Ried et al., 2012; Struski et al., 2002). Cervical cancers often display a gain of chromosome arms 3q and 5p, while colon carcinomas frequently gain chromosome 7 and lose chromosome 18 (Ried et al., 2012; Tsafrir et al., 2006). While many of these so called aneusomies are tissue-specific, some also occur more broadly regardless of cell type. Gains of chromosome 8q or 17q and loss of 13q are for example common in different types of cancers (Struski et al., 2002). Chromosomal aberrations are thus highly associated with cancer and are believed to contribute to malignancy. The seemingly random set of aneuploid chromosomes that appear in cancer cells may therefore be a product or endpoint of a chromosomal evolution in which a neoplastic chromosome combination has evolved (Duesberg et al., 2005). Many karyotypes of malignant cancers have near or above triploid chromosome numbers and a "genomic convergence" toward 3N may offer optimal redundancy of one spare chromosome for each pair of normal chromosomes (Chiba et al., 2000; Duesberg et al., 2005; Heim et al., 1988).

CGH and cDNA microarray analysis have been used to investigate how alterations in DNA copy number affect global gene expression in different types of tumor tissues. Over 60% of highly amplified genes also show elevated expression in human breast tumors and breast cancer cell lines (Pollack et al., 2002). In this study, recurrent regions of DNA copy number gain and loss were easily recognized, most of which had been previously linked to breast cancer (Forozan et al., 2000; Kallioniemi et al., 1994). The total number of genomic alterations was also found to be much higher in tumors of high grade or in tumors that had mutations of p53. The same correlation between severe aneuploidy and high grade metastatic tumors was established in squamous cell carcinomas of the head and neck (HNSCC) (Soder et al., 1995). In colorectal carcinomas and prostate tumor cell lines the acquired chromosomal aneuploidies modify global gene expression in a similar manner. There is a clear association of downregulated genes with regions of DNA loss and upregulated genes with regions of DNA gain (Phillips et al., 2001; Ried et al., 2012; Tsafrir et al., 2006; Wolf et al., 2004). In addition, the transcriptional activity of genes residing on unaffected chromosomes is probably also altered, leading to complex deregulation of the whole transcriptome. Such an outcome has been shown irrespective of the altered chromosome (Ried et al., 2012; Stingele et al., 2012; Torres et al., 2010).

These studies show that the relationship between aneuploidy and cell behavior is very complex and that context matters when determining what pathways and functions are

affected (Baker et al., 2009; Sotillo et al., 2009). Cell type, genetic background, population size, chromosome losses vs. gains and mechanisms of generating the aneuploidy all influence the probability of producing a cell that is able to survive with chromosomal defects and/or become tumorigenic (Weaver & Cleveland, 2008; 2009; Holland & Cleveland, 2009).

3.3 Aneuploidy and cancer

During the last ten years, there has been a vast amount of research dedicated to elucidating the causal link between aneuploidy and cancer. Most of these studies have focused on defects in the SAC, cytokinesis or other aspects of the cell division machinery.

3.3.1 Mitotic defects associated with aneuploidy and cancer

3.3.1.1 Spindle assembly checkpoint

Complete loss of SAC components causes embryonic lethality due to massive chromosome missegregation. On the other hand, partial loss of for example Mad2 function leads to premature sister-chromatid separation which causes a high degree of aneuploidy and polyploidy in MEFs and in human cancer cells. Heterozygous $Mad2^{+/-}$ animals develop lung tumors at high rates after long latencies (Michel et al., 2001). Mice heterozygous for the checkpoint protein Mad1, which binds to Mad2, also develop tumors in various tissues and display increased aneuploidy in MEFs (Iwanaga et al., 2007). Heterozygosity of the mitotic checkpoint protein CENP-E (centromere-associated protein-E) acts both oncogenically and as a tumor suppressor. MEFs with reduced levels of CENP-E become aneuploid due to inability of one or a few chromosomes to make stable attachments to the spindle microtubules (Weaver et al., 2007). This enhances the ability to form foci on plastic in primary and immortalized MEFs. It also facilitates anchorage-independent growth in soft agar. In addition, the cells that were able to grow in soft agar formed tumors when propagated *in vitro* and injected into mice. Conversely, aneuploidy caused by CENP-E heterozygosity also has a protective function against tumors induced by the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) or by loss of the tumor suppressor gene p19/ARF. $CENP\text{-}E^{+/-}$, $p19/ARF^{-/-}$ animals display significantly longer tumor latency compared to $p19/ARF^{-/-}$ animals with normal CENP-E function (Weaver et al., 2007). One explanation for this phenomenon is that aneuploid cells that already have high levels of CIN become sensitive to the DNA damage induced by the loss of tumor suppressor genes or gains of oncogenes. That is, cells would be able to survive the strain of some DNA damage or CIN, but a combination of these would raise the level of genetic instability and provoke cell death and tumor regression (Weaver et al., 2007).

Heterozygosity of SAC proteins does not always result in an increase in the formation of spontaneous tumors in animals, although MEFs derived from such animals may often show increased levels of aneuploidy (Baker et al., 2004; Baker et al., 2006; Jeganathan et al., 2007; Kalitsis et al., 2005). However, in almost all cases where reduced expression of a mitotic checkpoint protein leads to aneuploidy increased

susceptibility to carcinogen-induced tumor formation is nevertheless detected (Holland and Cleveland, 2009). Interestingly, causing CIN and aneuploidy via deregulation of the SAC component Bub1 has been shown to lead to loss of heterozygosity of tumor suppressor genes resulting in increased tumor formation. However, this increase was only seen in specific genetic backgrounds, i.e. in $p53^{+/-}$ and $APC^{Min/-}$ mice, but not in $PTEN^{+/-}$ or $Rb^{+/-}$ mice (Baker et al., 2009), emphasizing context-dependency. Loss-of-function mutations of SAC genes are quite infrequent in cancer, but decreased and especially increased expression of SAC proteins is more commonly detected (Cahill et al., 1998; Weaver and Cleveland, 2006). Mad2 overexpression can delay exit from mitosis and thus cause non-disjunction, where both sister chromatids in a pair are pulled to the same pole, resulting in aneuploidy (Hernando et al., 2004). Mad2-overexpressing mice develop a wide range of lethal tumors and it is thought that the aneuploidy and genomic instability resulting from this mitotic defect is the main initiator of tumorigenesis (Sotillo et al., 2007). Interestingly, increased levels of Mad2 has a more severe effect on tumor formation compared to decreased amounts, which could be due to the chromosome breaks and amplifications detected in transgenic mice overexpressing Mad2. Furthermore, Mad2 overexpressing cells often fail to execute cytokinesis and become tetraploid, which could be a route to aneuploidy and subsequently tumorigenesis (Sotillo et al., 2007). p53 and Rb have been shown to control the expression of Mad2 and upregulation of Mad2 in cells lacking Rb and p53 is necessary for induction of aneuploidy which accelerates transformation (Hernando et al., 2004; Schvartzman et al., 2011).

3.3.1.2 Other mitotic proteins

In addition to the SAC proteins, defects in other aspects of mitosis have also been shown to influence cancer formation. Mice heterozygous for the mitotic protein Cdc20 have a significantly increased rate of tumor formation compared to wild-type mice. An inactivating mutation of Cdc20 renders the mitotic checkpoint dysfunctional, which results in aneuploidy in MEFs (Li et al., 2009). In these homozygous Cdc20 mutant MEFs, as well as in other aneuploid mouse cells generated by a defective SAC, activation of the kinase ATM and its downstream target p53 was detected (Li et al., 2010). These cells have increased formation of reactive oxygen species (ROS) due to the high energy consumption associated with aneuploidy (Torres et al., 2007; Williams et al., 2008). The ROS could either directly, or by causing an increase in oxidative DNA damage, activate ATM and subsequently p53 resulting in cell death. p53 is thus speculated to function in an "aneuploidy checkpoint", as depleting ATM, p53 or ROS improves the growth of the cells and their ability to transform (Li et al., 2010). Many tumor types, including Ewings' sarcomas, glioblastomas and melanomas, share a genomic deletion or loss of expression of STAG2, a subunit of the protein complex cohesin that maintains cohesion between the sister chromatids. STAG2 deficient cells show a defect in sister chromatid cohesion leading to aneuploid cell divisions and a wide range of chromosome numbers. This phenotype was also verified by introducing a nonsense mutation in STAG2 in the near-diploid stable cell line HCT116 (Solomon et al., 2011). MEFs deficient in the SA1-containing cohesin complex also show chromosome segregation errors and aneuploidy due to incomplete telomere replication.

This could explain the early onset tumorigenesis detected in SA1 heterozygous mice (Remeseiro et al., 2012). In addition, high levels of proteins involved in dissolving sister chromatid cohesion have been found in different cancers and overexpression of for example separase and securin lead to aneuploidy *in vitro* (Yu et al., 2003; Zhang et al., 2008a).

3.3.1.3 Centrosome abnormalities

The correct segregation of chromosomes in mitosis is highly dependent on the centrosomes. The presence of multiple centrosomes in tumors has been known for a long time, although its link to transformation has been less evident. A centrosome consists of two centrioles embedded in the pericentriolar matrix. Centrosome duplication occurs during S phase and several protein kinases presumably have a role in this process. One of them is Polo-like kinase 4 (Plk4) and overexpression of Plk4 drives centrosome overduplication (Habedanck et al., 2005). This has been shown to lead to abnormal cell division in *Drosophila* neural stem cells, which normally divide asymmetrically, and brain tissue from Plk4-overexpressing mutant flies transplanted into wild-type hosts formed tumors in ~20% of the cases (Basto et al., 2008). Structural and numerical centrosome aberrations have also been found in different squamous cell carcinomas, as well as pancreatic, breast and prostate cancer (Gustafson et al., 2000; Lingle et al., 2002; Pihan et al., 2001; Sato et al., 1999; Thirthagiri et al., 2007), where they are often linked to chromosomal instability. As the presence of extra centrosomes could lead to a chaotic mitosis, cells can protect themselves by allowing clustering of the centrosomes using molecular motors and thus the formation of a normal bipolar spindle (Quintyne et al., 2005). However, the occurrence of merotelic attachments could be a reason behind the chromosomal instability in tumors containing extra centrosomes (Ganem et al., 2009; Silkworth et al., 2009). Although multiple centrosomes could be the result of abnormal centrosome duplication, the existence of excess centrosomes in cells is in the majority of cases the result of an abortive cell cycle.

3.3.2 Polyploidy, aneuploidy and cancer

Generation of polyploidy may also play a role in cancer formation. Tetraploidy or near-tetraploidy has been detected in early-stage cancers, for instance in Barrett's esophagus and cervical cancer (Galipeau et al., 1996; Olaharski et al., 2006). Multinucleate polyploid cells are therefore thought to act as genetically unstable intermediates that promote tumorigenesis by the formation of aneuploid daughter cells.

Maintaining functional cytokinesis is important in diploid mouse ovarian surface epithelial cells (MOSECs), a cell line that is spontaneously immortalized and transformed by prolonged culturing. Through fluorescence in situ hybridization (FISH), DNA FACS and chromosome counting the ploidy of the MOSECs was determined form early to late stage passaging. At early stages the population was mainly diploid while the proportion of tetraploid cells increased with cell passaging, reaching 51% of the population at passage 26 (p26) and decreasing to 28% at p36, as determined by FISH. Interestingly, the presence of near-tetraploid aneuploid cells

followed the increase in tetraploid cells, reaching its peak at p36 when more than 2/3 of the population was aneuploid. The tetraploid cells were derived from diploid cells failing to execute cytokinesis, although the reason for this failure was not determined. The tetraploid cells consequently gave rise to aneuploid cells through either multipolar mitoses or bipolar mitosis with missegregation events (Lv et al., 2012). Importantly, late passage aneuploid MOSECs produce tumors when injected into mice.

Loss of the tumor suppressor p53 facilitates the formation of tetraploidy in some cell types and is often required for tumor formation in mutated cells with genomic instability (Andreassen et al., 2001; Livingstone et al., 1992). Tetraploidy can be induced in p53-deficient mouse mammary epithelial cells (MMECs) by transiently blocking cytokinesis (Fujiwara et al., 2005). Although these cells were genetically unstable, formation of tetraploidy alone was not enough to induce transformation in culture. However, tetraploid MMECs seem to be more sensitive to carcinogens, as these cells showed anchorage-independent growth in soft agar following treatment with a mutagen. Without carcinogen treatment, these tetraploid cells produced tumors with a latency of 9-12 weeks when injected subcutaneously into nude mice. Cultures derived from both diploid and tetraploid $p53^{-/-}$ MMECs develop whole-chromosome aneuploidy, but the proportion of aneuploidy and gross chromosomal rearrangements is higher in tetraploid-derived cells. This is possibly due to multiple centrosomes and multipolar mitoses (Fujiwara et al., 2005). In cultured human colon carcinoma HCT 116 cells induced to tetraploidy through cytokinesis failure, it was shown that absence of p53 also is permissive for multipolar asymmetric divisions to occur. Multipolar mitoses are more frequent in these cells in the presence of the Mos oncogene (the cellular homologue of v-Mos from Moloney murine sarcoma virus). Mos prevents the coalescence of centrosomes, suggesting a mechanism for the oncogenic function of Mos (Vitale et al., 2010). The fate of tetraploid Swiss 3T3 mouse fibroblasts, induced by cell fusion or transient cytokinesis failure, also depends on the presence or absence of p53. p53 proficient cells either disappear from the population or propagate stably as tetraploids during many generations. Conversely, depletion of p53 rendered these tetraploid cells chromosomally unstable and prone to transformation (Ho et al., 2010).

Plk4 is essential for centriole duplication and it was recently shown that $Plk4^{+/-}$ MEFs exhibit a high rate of cytokinesis failure because of disruption of RhoA, which is needed for proper localization of the cleavage furrow (Rosario et al., 2010). This leads to multinucleation and supernumerary centrosomes and after several passages in culture, these cells are immortalized, acquire chromosomal abnormalities and also form tumors when injected into mice. Loss of heterozygosity of Plk4 is common in human hepatocellular carcinomas and may thus play a role in tumor initiation (Rosario et al., 2010). In addition to its role in β-catenin signaling, the tumor suppressor APC is also involved in microtubule stabilization and spindle checkpoint activation. It has been shown that a truncated form of APC causes failure of cytokinesis in cultured cells because of unanchored spindles and blocking of the cleavage furrow initiation (Caldwell et al., 2007). Loss of APC altogether leads to tetraploidy both *in vitro* and *in vivo* due to mitotic slippage, possibly as the results of mitotic spindle defects and a

compromised spindle checkpoint (Dikovskaya et al., 2007). This link between APC mutations and tetraploidy could be early events in the formation of colorectal cancer (Caldwell et al., 2007). Also, binucleated MCF10A cells generated as a result of entosis display both bipolar and tripolar mitosis and give rise to aneuploid progeny (Krajcovic et al., 2011). Cell-in-cell structures correlates with high grade breast tumors (Abodief et al., 2006). In colorectal cancer cell lines and Wilms tumor cells multipolar cell divisions is coupled to failed cytokinesis, which subsequently generates trisomic and tetrasomic daughter cells (Gisselsson et al., 2010).

Several other proteins deregulated in cancer have been shown to have a role in cytokinesis. For example loss of the transcription factor GATA6 is common in ovarian cancer and leads to mitotic defects and formation of tetraploidy in human ovarian surface epithelial cells (Cai et al., 2009; Capo-chichi et al., 2009). Increased activity of mitotic kinases have been described to induce multinucleation and polyploidy. Among them are Aurora A, Aurora B and Plk1 which are overexpressed in a variety of cancers (Fu et al., 2007; Incassati et al., 2006). Overexpression of c-Myc has been shown to induce spontaneous tetraploidization when p53 is inactivated (Yin, 1999) and the DNA damage resulting from telomere shortening, an event associated with early tumor formation, can induce tetraploidy (Davoli et al., 2010; Davoli and de Lange, 2012). Deregulation of many proteins and signaling pathways consequently lead to polyploidy, several of which are found in cancer cells.

AIMS OF THE STUDY

Although much progress has been made in elucidating the underlying causes behind cancer, many questions still need answering. Aneuploidy is prevalent in cancer, but its role in transformation is still unclear and the actual *in vivo* causes of aneuploidy are not known. Furthermore, detailed knowledge on how the effects of aneuploidy are influenced by genetic context and the microenvironment as well as how chromosomal deviations contribute to tumorigenesis on a molecular level is still limited. In order to improve early detection of malignant cells and target the spread of cancer it is essential to identify the triggers of oncogenic transformation and molecules that enable characteristics such as increased motility. The aim of this study was to acquire a deeper knowledge on the role of cell adhesion receptors in the development of cancer and in cancer cell invasion.

The specific aims of this study were:

- To detemine the role of integrin traffic in the generation of aneuploidy and tumorigenesis *in vitro* and *in vivo.*
- To elucidate the specific signaling changes contributing to the malignant phenotype of aneuploid cells.
- To study the role of integrins and syndecans in matrix remodeling and K-Ras-driven breast cancer cell invasion.

MATERIALS AND METHODS

More detailed information on the materials and methods is available in the original publications.

Methods

Method	Used in
Apoptosis assay	I
Array comparative genomic hybridization	I, II
Cell culture	I, II, III, unpublished
Cell fractionation	II
Chromosome spreads	I, II
Collagen contraction	III
Endocytosis assay	I
Enzyme linked sorbent assay	II
Flow cytometry	I, II, III
Fluorescence in situ hybridization	I
Foci formation assay	I, II
Hyaluronan secretion assay	II
Illumina gene expression	II
Immunofluorescence imaging	I, II, III, unpublished
Immunohistochemistry	I, II
In vivo experiments	I
Ingenuity Pathway Analysis	II
Invasion in Matrigel and Collagen	I, II, III, unpublished
Proliferation assay	I, II, III, unpublished
qRT-PCR	II, III, unpublished
RT^2 PCR Array	II
Soft agar assay	I
Statistical analysis	I, II, III, unpublished
Time-lapse imaging	I
Western blot	I, II, III, unpublished

Additional methods used in the study

Mouse phospho-receptor tyrosine kinase array
The mouse phospho-RTK array was used according to the manufacturer's protocol. Briefly, MEFs were lysed with lysis buffer provided by the Mouse phospho-RTK kit (R&D Systems) and protein concentration was measured. Nitrocelullose membranes containing 39 different anti-RTK antibodies printed in duplicate were incubated with equal amounts of cell lysate from each sample, washed, and incubated with a HRP-conjugated pan anti-phospho-tyrosine antibody. Phosphorylated RTKs were detected with enhanced chemiluminescence (ECL) and analyzed using ImageJ.

Immunoprecipitation
MEFs were lysed in 40 mM Hepes NaOH (pH 7.5), 75 mM NaCl, 2mM EDTA, 1% NP-40 and protease and phosphatase inhibitor cocktails (Complete and PhosStop; Roche) and lysates were incubated with EphA2 antibody bound to protein G–Sepharose beads. Immunoprecipitates were washed three times with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1% NP-40 and precipitated proteins were detected with western blotting analysis with EphA2 and the anti-phospho-tyrosine-HRP antibody from the phospho-RTK array kit.

DNA constructs

- H-Ras V12 from Michael Karin (University of California, San Diego, La Jolla, CA) (I)
- pcDNA-Flag Twist2 from Li Li (Center for Mol. Medicine & Genetics, Detroit, MI) (II)

siRNAs

- Rab21: 5'AAGGCATCATTCTTAACAAAG 3' (Qiagen) (I)
- Scr: All Stars Neg. Control (1027281, Qiagen) (I, II, III)
- Twist2: siGenome SMARTpool M-044881-00-0005 (Dharmacon) (II)
- Twist2: D-044881-01 siGENOME siRNA target sequence GCGACGAGAUGGACAAUAA (Dharmacon) (II)
- Twist2: D-044881-04 siGENOME siRNA target sequence CCGCCAGGUACAUAGACUU (Dharmacon) (II)
- Syndecan-1: (Beauvais et al., 2004) (III)
- Syndecan-4: (Rauch et al., 2005), (III)
- MT1-2-3-MMPs: SI03648841: 5'CAGCGATGAAGTCTTCACTTA 3' ; SI00037688; SI00083006: 5'AAGCACATCACTTACAGTATA 3' (Qiagen) (III)
- K-ras: SI02662051 (Qiagen) (III)
- K-ras: 4390824: 5'GCCUUGACGAUACAGCUAAtt 3' (Ambion) (III)
- Eps8: 5'GCCATGCCTTTCAAGTCAACTCCTA 3' (Invitrogen) (unpublished)
- EphA2: FlexiTube siRNA SI00994651: 5' ACCCATGATGATTATCACAGA 3' (Qiagen) (unpublished)

Cells

Cells	Description	Used in
MEF	Murine embryonic fibroblast	I, II, unpublished
MDA-MB-231	Human breast adenocarcinoma	III
CHO	Chinese hamster ovary	III
HMEC	Human mammary epithelial cell	I

Antibodies

Target	Description	Application	Used in
Actin	anti-mouse (Sigma)	WB	unpublished
CD44	Purified NA/LE Rat anti-mouse (BD Pharmingen)	Blocking	II
EphA2	C-20 sc-924 rabbit polyclonal (Santa Cruz)	IF, WB, IP	unpublished
Eps8	610143 Mouse IgG1 (BD Transduction Laboratories)	WB, IF	unpublished
Has1	sc-34021 (Santa Cruz)	IF	II
Histone H3	(D1H2) XP ® Rabbit mAb (Cell Signaling Technology)	WB	II
phospho-FAK	Tyr397, 44-624G (Invitrogen)	IF, WB	III, unpublished
phospho-myosin light chain	pS19, ab4720 rabbit polyclonal (Abcam)	IF, WB	III
phospho-p44/42	Thr 202/Tyr 204, 9101, rabbit, (Cell Signaling Technology)	WB	unpublished
phospho-Src	Tyr416, 2101, rabbit (Cell Signaling Technology)	WB	unpublished
phospho-tyrosine-HRP	from mouse phospho-RTK array kit (R&D Systems)	IP	unpublished
Syndecan-1	mAb B-B4 (AbD Serotec)	IF, FACS	III
Syndecan-4	AF2918 (R&D Systems).	IF, FACS	III
Twist2	Mouse monoclonal ab57997 (Abcam)	IF, IHC,WB	II
α2 integrin	mAb MCA2025 (AbD Serotec)	IF	III
α2 integrin	mAb P1H5 (Santa Cruz)	Blocking	III
α7 integrin	anti-mouse, (3C12) (MBL International Corporation)	IF, FACS	II

α7 integrin	anti-mouse, (6A11) (MBL International Corporation)	Blocking	II
α-tubulin	mouse, 12610 (Hybridoma bank)	WB	I, II, III, unpublished
β1 integrin	Rat monoclonal, Mab1997 clone MB1.2 (Millipore)	FACS	I
β1 integrin	LEAF purified anti-mouse/rat CD29 clone HMβ1-1 (Biolegend)	Blocking	I
β1 integrin	K20 (Beckman Coulter)	Blocking	I
β1 integrin	EP1044, rabbit monoclonal (Transduction Laboratories)	WB	I
γ-tubulin	rabbit, 11317 (Abcam)	IF	I

Abbreviations: FACS, *flow cytometry*; IF, *immunofluorescence*; IHC, *immunohistochemistry*; IP, *immunoprecipitation*; WB, *Western blot*

Reagents

Reagent	**Manufacturer/Supplier**	**Application**	**Used in**
Acid-extracted rat tail collagen I	Sigma-Aldrich	Invasion, IF	III
Apo-ONE	Promega Corporation	Apoptosis	I, unpublished
Cycloheximide	Sigma-Aldrich	Apoptosis	I
Dabco	Sigma-Aldrich	IF	I, II, III, unpublished
DAPI	Sigma-Aldrich	IF	I, II, III, unpublished
Dasatinib	Selleck	Proliferation, invasion, WB	unpublished
DRAQ5	Biostatus Ltd	IF	II
Growth factor reduced Matrigel	BD Biosciences	Invasion	I, II
Hematoxylin & eosin	Sigma-Aldrich	Tissue staining	I, III
Hiperfect	Qiagen	Transfection	I, II,unpublished
Laminin	Sigma-Aldrich	Dish coating	I
Lipofectamine 2000	Invtrogen	Transfection	I, II, III
MMP inhibitor GM6001	Calbiochem	Invasion	III
Mowiol	Calbiochem	IF	I, II, III, unpublished
Pepsin-extracted collagen I	Advanced Biomatrix/ Inamed Biomaterials	Dish coating, contraction, invasion, IF	III

Phalloidin	Molecular Probes	IF, invasion	I, II, III
PP2	Merck Chemicals Ltd	Proliferation, invasion	unpublished
Protein G-sepharose beads	Amersham Bioscicences	IP	unpublished
ROCK inhibitor Y27632	Sigma-Aldrich	Contraction	III
TNF-α	Peprotech Inc.	Apoptosis	I
Vectashield Mounting medium	Vector Labs	IF	I, II, III, unpublished
WST-1	Roche Applied Science	Proliferation	I, II, III, unpublished

Abbreviations: IF, *immunofluorescence*; IP, *immunoprecipitation;* WB, *Western blot*

Animals

8 weeks old female athymic nude mice were used for the *in vivo* xenograft experiments (I).

RESULTS

1. Failed integrin traffic induces aneuploidy and cell transformation (I, II)

Polyploid cells have been detected in the early stages of particularly cervical cancer (Olaharski et al., 2006), and the formation of unstable multinucleate cells is consequently thought to function as an intermediate state in the formation of aneuploid malignant cells. Integrin traffic has been shown to be important for the proper execution of cytokinesis and inhibition of this traffic leads to a binucleate phenotype (Pellinen et al., 2008). Furthermore, a genetic deletion of the small GTPase Rab21, which regulates the endo-and exocytic traffic of β1 integrin, leads to binucleation in cultured KFr13 ovarian carcinoma cells (Pellinen et al., 2008). To investigate the potential role of integrin traffic in the formation of aneuploidy we silenced Rab21 in normal human mammary epithelial cells (HMECs) for a period of three weeks. This led to the accumulation of an increased amount of bi- and multinucleate cells compared to scrambled siRNA (Scr) control cells (I, Figure 1c), consistent with previous findings. Furthermore, two weeks after the RNAi treatment ended there was a clear induction of aneuploidy in the Rab21 silenced cells, where 16/17 cells displayed an aneuploid karyotype. In contrast, the majority of Scr control siRNA transfected cells had remained diploid with a chromosome number of 46 (I, Figure 1d). Impairing integrin traffic through downregulation of Rab21 is thus sufficient to induce aneuploidy in human cells. Interestingly, we also found that loss of Rab21 correlates with increased malignancy in prostate and ovarian carcinoma samples (I, Figure 1a, b), possibly reflecting a role for Rab21 in maintaining cell ploidy.

To further study if impaired integrin traffic could lead to malignant transformation, we employed mouse embryonic fibroblasts (MEFs) that harbor a germline mutation in the NPXY motifs of their β1-integrin. This tyrosine to phenylalanine mutation (β1YY783, 795FF) causes reduced clathrin-mediated endocytosis of the integrin (I, Figure S1a), which allowed us to study the consequences of impaired integrin traffic. Two clones (a and b) of wild-type and β1YYFF MEFs were passaged on the β1-specific matrix component laminin and underwent one cell division cycle. This led to failed cell division of the β1YYFF cells due to their dependency on the β1-subunit on this matrix. All cells were subsequently grown to confluency under conditions were adhesion was mediated via other integrin subunits, allowing normal cell division to take place. This passaging on laminin was repeated either 4 times (for both clones) or 8 times (for clone a only) and all surviving cells were subsequently passaged under normal conditions (I, Figure 2b; II, Figure 1a), after which their phenotypes were analyzed. Interestingly, the repeated cycles of cytokinesis failure led to the acquisition of an aneuploid, near-triploid, karyotype in both β1YYFF_L4 and β1YYFF_L8 cells (the number of passages on laminin are indicated with L0, L4 and L8) (I, Figure 2c; II, Figure 1b). This was assessed by chromosome counting of metaphase spreads. The wild-type cells,

however, were mostly unaffected by the treatment and displayed modal chromosome numbers of 80 (I, Figure 2c; II, Figure 1b). These MEFs have been immortalized with SV40 Large T which leads to spontaneous tetraploidization of the genome, resulting in 80 chromosomes (Weaver et al., 2007). However, neither β1wt nor β1YYFF MEFs have any apparent phenotype when cultured under standard conditions.

We continued to study the phenotype of the a clone (here on referred to only as β1YYFF) of the aneuploid MEFs, with a particular interest in characteristics associated with malignancy. We found that the β1YYFF_L4 cells had regained the ability to endocytose β1-integrin (I, Figure 4c), probably via a clathrin-independent pathway, and were thus able to divide on β1-specific matrixes (I, Figure 4a). However, consistent with previous reports on the reduced fitness associated with aneuploidy, the aneuploid L4 and L8 cells proliferated more slowly under adherent conditions compared to tetraploid cells (I, Figure S3b; II, Figure 1c). In contrast, we found that these cells are significantly more able to proliferate under conditions that do not require anchorage to a substrate, a well-known hallmark of cancer cells (I, Figure 3a; II, Figure 1d, S1a, b). This was studied by allowing the cells to grow in either the polysaccharide agarose or on ultra-low attachment plates. In addition, the aneuploid β1YYFF_L4 cells were more resistant to TNF-α-induced cell death and growth factor deprivation compared to tetraploid control MEFs (I, Figure 3b, c). The ability to migrate and invade into extracellular matrix is considered a property specific to transformed cells and associated with cancer cell metastasis. Intriguingly, the aneuploid β1YYFF_L4 and β1YYFF_L8 cell populations were significantly more invasive in the reconstituted basement membrane matrix Matrigel than tetraploid control cells (I, Figure 4d; II Figure 1e). Failed integrin traffic and the sequential acquisition of aneuploidy are thus linked to many features associated with cancer. Importantly, the aneuploid cells were also able to form highly malignant and fast-growing tumors upon subcutaneous injection into mice (I, Figure 5a). The non-transformed tetraploid β1YYFF_L0 mutant MEFs also developed small tumors in mice, albeit with a much longer latency (I, Figure 5a). Interestingly, analysis of the xenografts revealed an aneuploid karyotype in the tumor cells originating from both the aneuploid β1YYFF_L4 cells as well as the tetraploid β1YYFF_L0 cells (I, Figure 5c, e). These results thus suggest that deregulated integrin traffic could induce aneuploidy and transformation *in vivo* as well.

2. Aneuploidy leads to molecular alterations (I, II)

Having established that these aneuploid cells are indeed transformed, we were interested in the molecular changes that have evolved due to the aneuploidy. This was done by analyzing gene copy number changes as well as transcriptional and proteomic alterations in the cells. We performed a multi-color fluorescence in situ hybridization (mFISH) analysis on six β1YYFF_L0 and six β1YYFF_L4 cells to get an overview of the karyotypic changes in the aneuploid cells. This analysis confirmed a variable number of chromosomes (from 53-83) in the cells within the β1YYFF_L4 population

(I, Figure 2d) and also variations in the copy number of individual chromosomes between cells. In addition, a subset of the chromosomes analyzed in the β1YYFF_L4 population displayed structural chromosomal abnormalities, such as translocations and fusions. However, array-based comparative genomic hybridization (aCGH) revealed only a few deletions or amplifications in β1YYFF_L4 (I, Figure S1b). Some chromosomes contained regions of copy number gain or loss, but the major chromosomal alterations in β1YYFF_L4 compared to β1YYFF_L0 seemed to be numerical.

An Illumina microarray-based genome-wide gene expression analysis of wild-type (L0, L4 and L8) and β1YYFF mutant (L0, L4 and L8) MEFs revealed that the aneuploid β1YYFF_L4 and β1YYFF_L8 cell populations had a very similar transcriptional profile (II, Figure 2a, b). However, when comparing the aneuploid β1YYFF_L4 and the tetraploid β1YYFF_L0 cells approximately 160 genes were differentially expressed in β1YYFF_L4, when the fold change threshold was ≥4 (II, Table S1). The differences between β1wt_L4 and β1wt_L0 were excluded from this analysis so as not to include gene expression changes that could have arisen as result of the laminin-treatment alone. Among the upregulated genes were many interesting genes previously linked to cancer, including *Has1* (hyaluronan synthase 1), *Eps8* (epidermal growth factor receptor substrate 8), *Twist2*, *Dusp4* (dual-specificity phosphatase 4), *Sirp-alpha* (signal-regulatory protein alpha) and *Itga7* (integrin alpha 7) (II, Table S1). Differential expression of some of these genes was also verified with qRT-PCR (II, Figure 2c). Ingenuity Pathway Analysis (IPA) was used to get a more comprehensive view of the signaling pathways affected by the aneuploidy. Interestingly, "Cancer" was the top biological function associated with the aneuploid cells. In addition, the β1YYFF_L4 cells showed an upregulation of many genes involved in the biosynthesis of steroids (II, Figure S2d). The IPA analysis also predicted increased lipid quantity and increased activity of the transcription factors *Srebf1* and *Srebf2*, the main regulators of fatty acid and cholesterol synthesis (II Figure S2c, e), suggesting an overall increase in lipid metabolism. microRNAs have also been shown to be involved in cancer progression, as they are important regulators of gene expression at the post-transcriptional level. We studied the microRNA profiles of β1wt_L0, β1wt_L4, β1YYFF_L0 and β1YYFF_L4 using an RT^2 miRNA Cancer pathway PCR Array and were able to find several aneuploidy-specific changes in miRNA expression (II, Figure S5c). For example miR-140, miR-146b and miR-10b were upregulated in β1YYFF_L4 compared to β1YYFF_L0 while miR-135b, miR-181d and miR-203 were downregulated. These analyses show that the aneuploid populations have acquired stable changes in their transcriptional profiles, as determined by studying both gene and microRNA expression.

Aneuploidy has been shown to have effects on the proteome and especially affect the stoichiometry of proteins involved in complexes. Receptor-tyrosine kinases (RTKs) are often deregulated in cancer cells and our intention was therefore to study both expression and activation of different RTKs in the aneuploid β1YYFF_L4 cells. Using a proteome profiler mouse phospho-RTK array that detects increased levels of

phosphorylated RTKs we found an increase in EphA2, EphA3 and M-CSF receptors in β1YYFF_L4 compared to the tetraploid β1YYFF_L0 (Figure 7). In contrast, the cancer –associated receptors Met, Axl and PDGFR showed decreased levels/activity in the aneuploid cells.

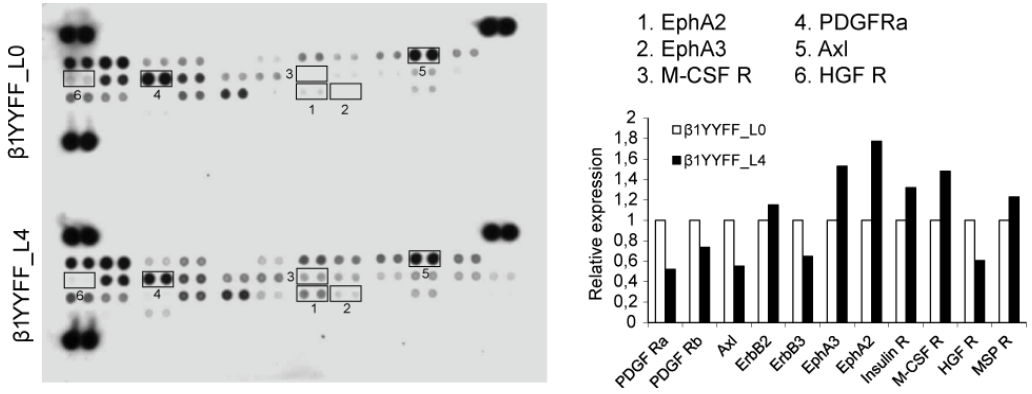

Figure 7. Differential expression of receptor tyrosine kinases. A proteome profiler mouse phospho-RTK array was used to determine tyrosine phosphorylation of RTKs in β1YYFF_L0 and β1YYFF_L4. The relative expression of RTKs in β1YYFF_L4 is normalized to β1YYFF_L0.

As the aneuploid cells were generated by inhibiting integrin traffic, we wanted to examine if signaling downstream of integrin activation is affected. Integrin activation was done by letting cells adhere to the β1-specific matrix collagen, whereas control cells were held in suspension only. To prevent serum proteins and growth factors from activating intracellular signaling pathways, serum-free medium was used. The samples were blotted with phospho-specific antibodies to detect the kinases Src, FAK and the extracellular signal-regulated kinases (Erk1/2) in their active state. The β1YYFF mutation has previously been shown to lead to decreased activation of FAK (Wennerberg et al., 2000) when studying signaling downstream of β1-integrin. Interestingly, the levels of active FAK were significantly higher in the aneuploid β1YYFF_L4 cells compared to β1YYFF_L0, both in adherent and suspended cells (Figure 8a) and similar differential activation could also be detected for Src (Figure 8a). These results indicate that there is a growth factor and integrin independent constitutive activation of Src and FAK in the aneuploid cells. The wild-type β1wt_L0 and β1wt_L4 cells showed similar levels of active Src and FAK in both adherent and suspended cells (data not shown). No significant difference was detected in the activity levels of Erk1/2 between the β1YYFF_L0 and β1YYFF_L4 cells (Figure 8a). Together, these results indicate that induction of aneuploidy has led to several molecular alterations, thus enabling a gain in expression and activity of several pathways involved in different cellular functions.

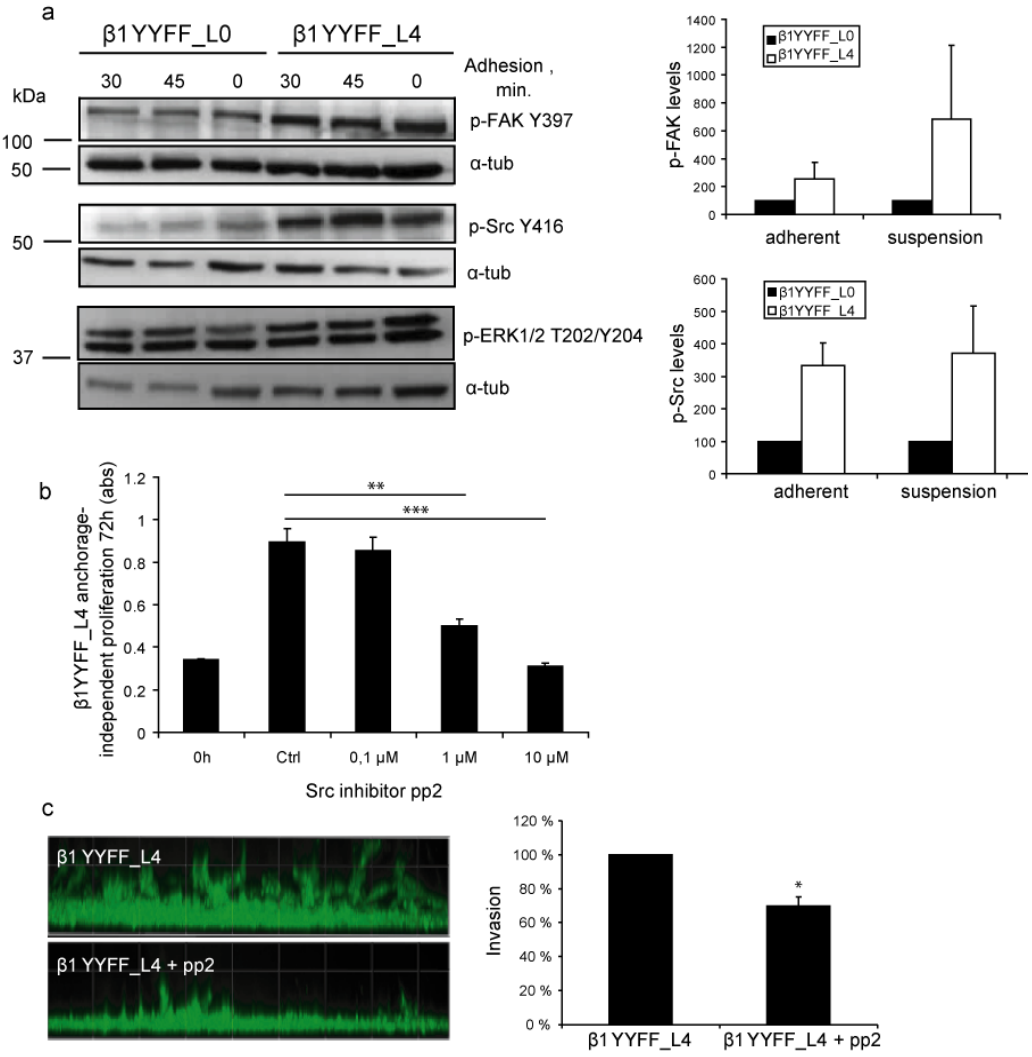

Figure 8. β1YYFF_L4 aneuploid cells have increased levels of active Src (a) β1YYFF_L0 and β1YYFF_L4 cells were held in suspension at 37ºC for 1h before plating on collagen I for 30 or 45 min. Lysates were probed for p-Src Y416 (60kD), p-FAK Y397 (125kD and p-ERK1/2 T202/Y204 (44/42 kD) and α-tubulin (55kD) (loading control)**.** Phospho-Src and p-FAK expression in adherent cells and cells in suspension is quantified (mean ± SEM, n = 3). (b) Anchorage-independent proliferation of β1YYFF_L4 in the presence of the indicated concentrations of Src inhibitor pp2 was measured after 72 h. Absorbance at 0h is shown as a reference (mean ± SEM, n = 4; **p<0.005, ***p<0.001). (c) 1 μM pp2 was added to the Matrigel. Representative side view images of control and pp2 treated β1YYFF_L4 cells after 4 days of invasion in Matrigel, 50 μm grid. Shown is an average from three independent experiments (mean ± SEM, n = 8-12; *p<0.05).

3. Distinct pathways contribute to the malignancy of aneuploid cells (II, unpublished)

By studying the aneuploidy-specific gene expression changes in more detail our aim was to identify molecules that have been linked to cancer and the various aspects of cancer cell signaling that distinguishes them from normal cells. The extracellular matrix component hyaluronan (HA) is known to trigger activation of RhoA and thus influence actin organization and motility in cancer cells (Bourguignon et al., 2006; Radotra and McCormick, 1997). *Has1*, one of the enzymes responsible for HA synthesis, and the muscle specific integrin alpha 7, *Itga7*, were the most upregulated genes verified with qRT-PCR (II, Figure 2c). We also confirmed increased surface expression of α7-integrin (II, Figure S4b) and increased Has1 and HA levels (II, Figure S3b, c) in the β1YYFF_L4 cells. However, loss-of-function experiments using siRNA-mediated silencing of these genes or function -blocking antibodies against α7-integrin or CD44, the receptor for HA, did not affect the ability of these cells to invade into Matrigel (II, Figure S3d, S4d; data not shown). We therefore focused our attention on another interesting gene, the transcription factor Twist2 which was upregulated 4.3-fold in β1YYFF_L4 compared to β1YYFF_L0 (II, Figure 3a). This protein has been linked to both EMT and apoptosis resistance. As determined by immunofluorescence and nuclear fractionation assays, Twist2 was mainly localized in the nucleus of the β1YYFF_L4 cells and its expression was also increased compared to the tetraploid cells (II, Figure 3c, d). Importantly, knockdown of *Twist2* significantly reduced the ability of the aneuploid cells to proliferate in suspension and also their invasiveness (II, Figure 3e-g). Thus, the Twist2 transcription factor clearly contributes to the aneuploid cells' proliferative capacity as well as motility. Twist2 is an important regulator of embryonal development and has also been linked to lipid production as the *Twist2* KO mice show adipose deficiency in skin, spleen and thymus (Sosic et al., 2003). We therefore analyzed the potential link between the upregulation of *Twist2* and the lipid biosynthetic pathway in the aneuploid β1YYFF_L4 cells. Interestingly, *Twist2* silencing led to decreased expression of many genes involved in cholesterol metabolism and lipoprotein signaling (II, Figure 4b), including several genes controlled by the Srebp1/2 transcription factors that were upregulated in the Illumina microarray (II, Figure 4a). Furthermore, inhibiting Srebp function by using the inhibitor Fatostatin resulted in reduced anchorage-independent growth, similarly to *Twist2* silencing (II, Figure 4c). It is thus possible that Twist2 elicits its pro-tumorigenic function via altering lipid metabolism, which is often detected in transformed cells (Clendening et al., 2010; Menendez and Lupu, 2007).

Among the upregulated genes in the aneuploid cells was also Dusp4 (II, Figure 2c), a close relative to Dusp1, which has been shown to mediate resistance to cisplatin-induced cell death via inactivating the apoptotic JNK pathway (Wang et al., 2006). Intriguingly, the aneuploid MEFs show increased resistance to cisplatin-induced apoptosis (Figure 9a). In addition, expression analysis from ovarian cancer cell lines show that KFr13 cells harboring a deletion of Rab21 have significantly higher levels of Dusp4 compared to the parental cell line KF28 (Figure 9b) and they also show

increased resistance to cisplatin treatment (Kikuchi et al., 1986). The two cell lines had similar expression levels of Dusp1 (Figure 9b). Dusp4 levels therefore seemed to correlate with cisplatin-resistance in both the aneuploid MEFs and KFr13 cells. The mechanism of cisplatin-resistance in these cells was not, however, studied further.

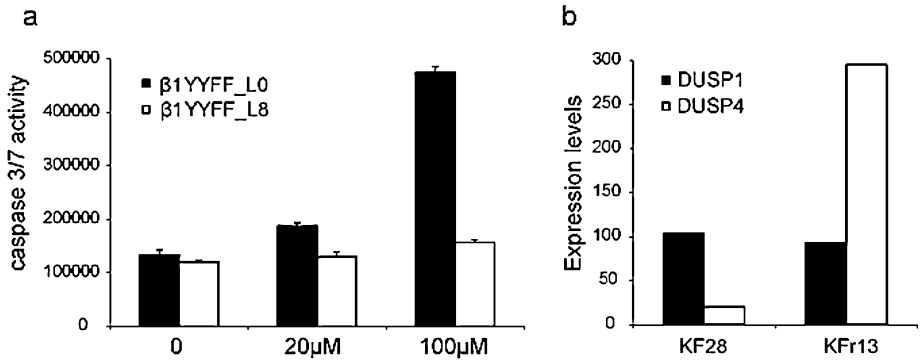

Figure 9. Aneuploid cells display increased resistance to cisplatin. (a) β1YYFF_L0 and β1YYFF_L8 cells were treated with cisplatin for 24 hours with the indicated concentrations. Apoptosis was measured using the Apo-ONE reagent. (b) Affymetrix expression levels of Dusp1 and Dusp4 in KF28 and KFr13 cells.

We were also intrigued by the increased activity of Src and FAK, as the Src/FAK pathway has been linked to among other things cancer cell invasion, survival and angiogenesis (Frame, 2004). Inhibition of Src kinase activity with the small molecule inhibitors PP2 or Dasatinib reduced anchorage-independent growth and invasion of β1YYFF_L4 cells (Figure 8b, c; data not shown). However, Src inhibition did not affect proliferation or migration under adherent conditions (data not shown), suggesting that these cells are dependent on Src signaling specifically in a 3-dimensional setting. Src kinase thus seems to contribute to the malignancy of the aneuploid cells but its activity is, however, not enough to cause transformation in tetraploid control MEFs. Overexpression of constitutively active Src alone was not sufficient to induce invasion of untransformed cells (data not shown).

Taken together, these results indicate that distinct pathways have evolved in the aneuploid cells to contribute to the transformed phenotype, promoting properties such as apoptosis resistance, anchorage-independent growth and invasion.

4. Src tyrosine kinase is regulated by Eps8 and EphA2 (unpublished)

As the significant upregulation of Src kinase activity and its downstream signaling clearly played a role in the malignancy of these cells, we were interested in the regulation of this pathway. As a starting point, we studied the gene expression changes

in the Illumina array in the search for differential expression of genes related to Src signaling. Eps8, which was upregulated ~5.8-fold in β1YYFF_L4 (II, Table S1), exerts both actin bundling and capping activity and promotes proliferation, migration and invasion in different types of cancer (Hertzog et al., 2010; Liu et al., 2010; Yap et al., 2009). Eps8 has also been proposed to both regulate Src activity and be under the control of Src (Liu et al., 2010; Maa et al., 2007). Therefore, we further investigated the potential link between the upregulation of Eps8 detected on the Illumina microarray and the constitutive activation of Src and FAK in the aneuploid cells. Eps8 upregulation was confirmed with both qRT-PCR (Figure 10a) and western blot analysis of protein expression (Figure 10b). Importantly, silencing of Eps8 in β1YYFF_L4 cells reduced invasion and anchorage-independent growth (Figure 10 c, d).

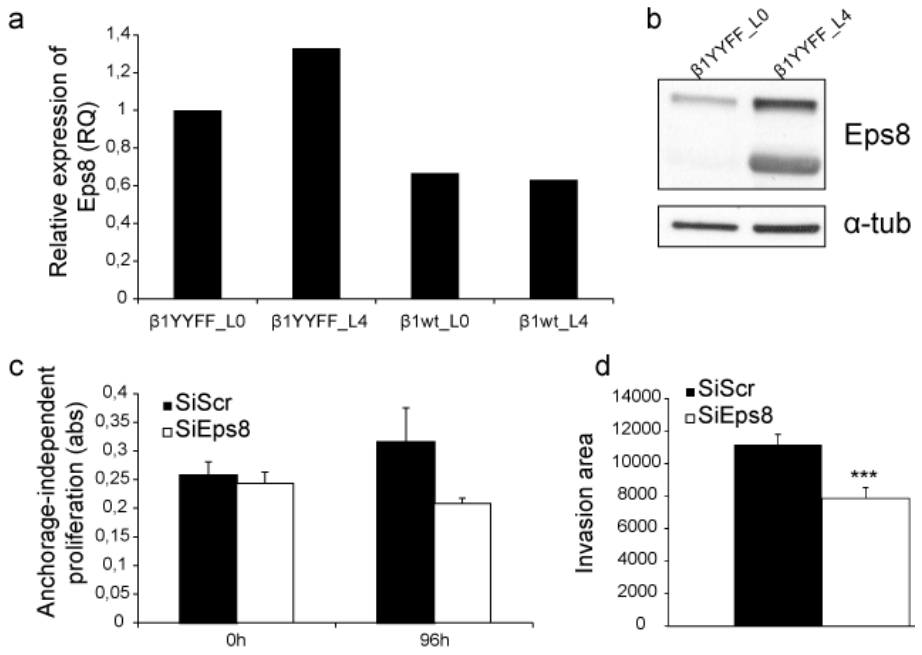

Figure 10. β1YYFF_L4 aneuploid cells have increased levels of Eps8. RNA (TaqMan qRT-PCR) (a) or protein (western blot, 68 kD and 97 kD) (b) of Eps8 levels in the indicated cell lines. TaqMan results were normalized using GAPDH as endogenous control. (c) Anchorage-independent proliferation of Scr silenced or Eps8 silenced β1YYFF_L4 cells (mean ± SEM, n = 4). (d) Invasion in Matrigel of Scr silenced or Eps8 silenced β1YYFF_L4 cells. Shown is an average from two independent experiments (mean ± SEM, n = 20; ***$p<0.001$).

v-Src has been shown to phosphorylate Eps8 and also increase Eps8 expression (Gallo et al., 1997). In the transformed β1YYFF_L4 MEFs, Dasatinib treatment effectively decreased the levels of active Src, but did not, however, alter Eps8 protein expression (Figure 11a). We therefore examined the effect of Eps8 knockdown on Src levels. As the difference in the levels of active Src and FAK between β1YYFF_L4 and β1YYFF_L0 was especially obvious in serum-starved cells held in suspension,

experiments were performed under these conditions. Silencing of Eps8 reduced the levels of pY416Src as well as pY397FAK, which was detected with antibodies specific for the autophosphorylation sites on the kinases (Figure 11b). pY416Src also had a more intracellular location in Eps8-silenced cells, as opposed to the more peripheral localization in control silenced cells (Figure 11c). Interestingly, p-Src and Eps8 often localized to the same area in the cell membrane in control silenced cells. Eps8 knockdown had, however, no detectable effect on the localization of pY397FAK as determined by immunofluorescence staining on adhered cells (Figure 11d).

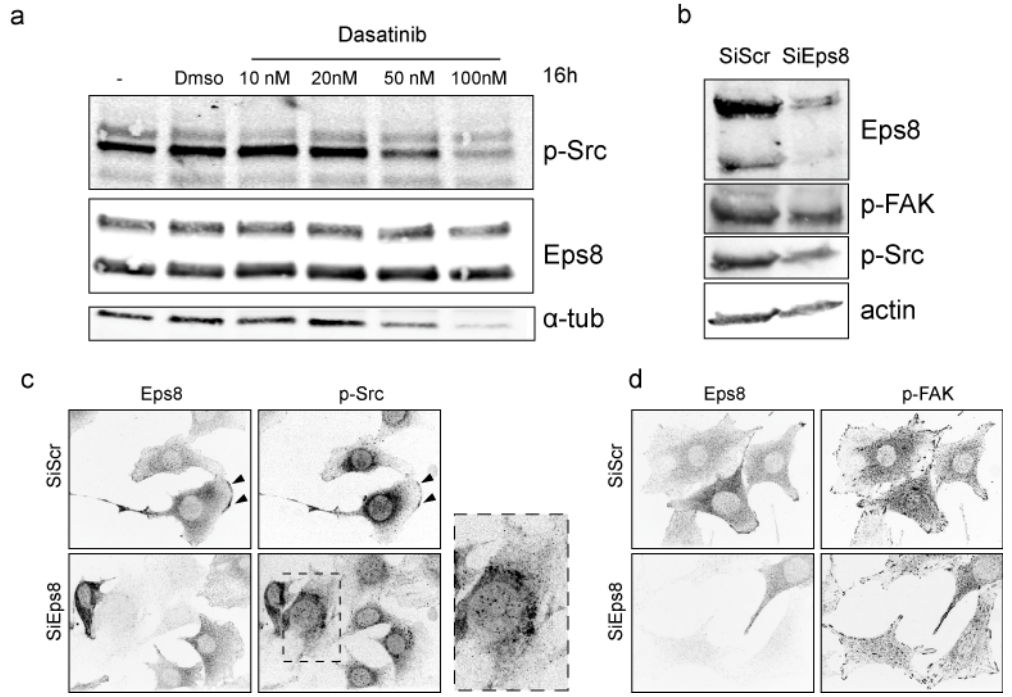

Figure 11. Src tyrosine kinase is regulated by Eps8 (a) Levels of p-Src, Eps8 and α-tubulin (loading control) after treatment with the indicated concentrations of Dasatinib for 16 hours. (b) Levels of p-Src, Eps8, p-FAK and actin (loading control) in Scr silenced or Eps8 silenced β1YYFF_L4 cells. Immunofluorescence images of Eps8 and p-Src (c) or Eps8 and p-FAK (d) in Scr silenced or Eps8 silenced β1YYFF_L4 cells. Arrowheads indicate localization of Eps8 and p-Src.

Src kinase is also activated downstream of RTKs and we therefore looked more closely at the data obtained from the proteome profiler phospho-RTK array. The EphA2 receptor has been shown to be required for Src-dependent invasion of colorectal cancer cells (Leroy et al., 2009) and Src kinase is known to act downstream of EphA2-mediated cell invasion (Faoro et al., 2010). In the aneuploid β1YYFF_L4 cells there seemed to be an increase in both expression and activity of EphA2, which was detected by immunoprecipitation of EphA2 and probing with a phospho-tyrosine specific antibody (Figure 12a). Interestingly, the overall tyrosine phosphorylation of β1YYFF_L4 was also increased compared to β1YYFF_L0, which could possibly

account for the elevated tyrosine phosphorylation detected in the immunoprecipitated EphA2 (Figure 12a). In addition to its perinuclear localization, Eps8 was mostly found at cell protrusions and was very often located in the same structures as EphA2 (Figure 12b). EphA2 knockdown did not seem to alter protein levels of active Src (data not shown), but did have an effect on its localization in the cell. Similarly to Eps8-silencing, EphA2 dowregulation in β1YYFF_L4 cells led to decreased localization of active Src at the membrane and accumulation in what looked like endosomal structures (Figure 12c).

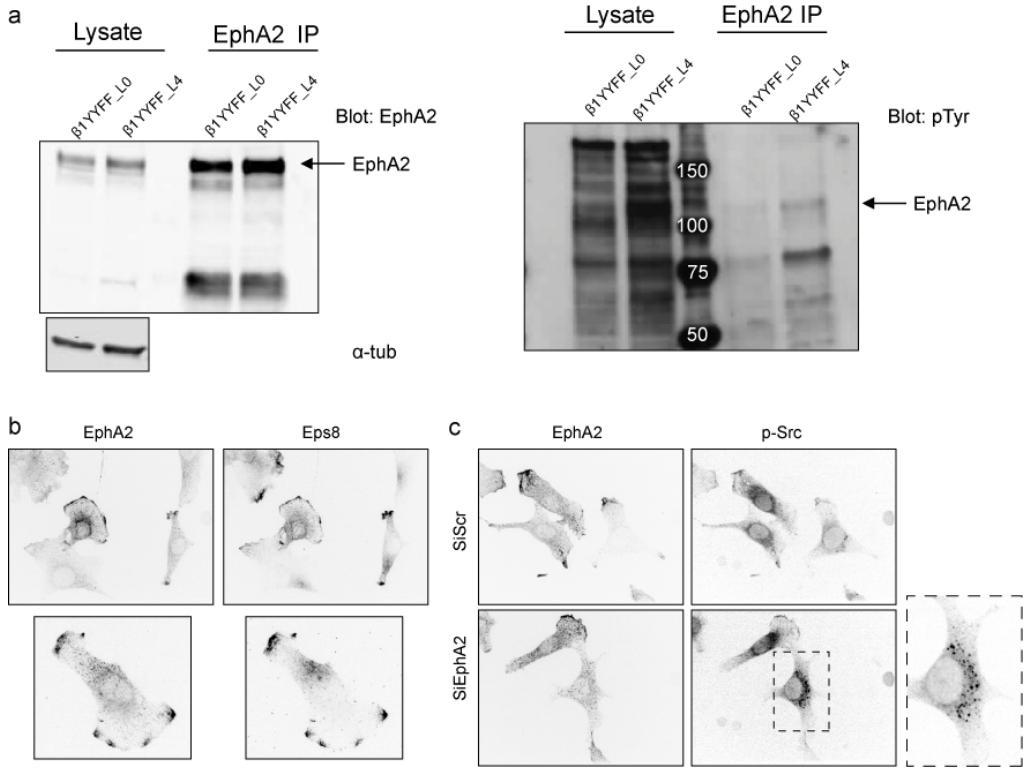

Figure 12. EphA2 regulates the location of p-Src (a) Immunoprecipitation of EphA2 blotted with antibodies against EphA2 or phospho-tyrosine. (b) Immunofluorescence images show similar localization of Eps8 and EphA2. (c) Immunofluorescence images of EphA2 and p-Src in Scr silenced or EphA2 silenced β1YYFF_L4 cells.

Eps8 upregulation and increased expression or activity of EphA2 thus seem to have been selected for to increase Src activation, leading to constitutive activation of this pathway independently of cell attachment or growth factor stimuli.

5. Syndecans and integrins regulate matrix contraction (III)

Matrix contraction and remodeling is necessary for the ability of cancer cells to invade through different matrixes and some cancer cells have been shown to employ CAFs to facilitate in the invasion. We assessed the different roles of the cell surface receptors integrins and syndecans in this process in highly malignant mesenchymal –like MDA-MB-231 breast adenocarcinoma cells. Cells seeded inside 3D pepsin-extracted collagen were efficiently able to contract the matrix during a 20 hour incubation (III, Figure 2a). This contraction was dependent on α2β1 integrin, since a function blocking antibody against α2 impaired the cells' ability to contract the matrix. By using stable GFP-syndecan-1 or -4 expressing MDA-MB-231 cells, we were also able to show that syndecan-1, and especially syndecan-4, were important for collagen contraction (III, Figure 2c). GFP-syndecan-4 cells increased contraction by as much as 45% compared to GFP cells after 24 hours incubation (III, Figure 2c). These results were also confirmed with MDA-MB-231 cells transiently overexpressing syndecan-1 and -4 (III, Figure 2c). Furthermore, at least syndecan-4 co-operates with α2β1 in matrix contraction, as blocking α2 function in GFP-syndecan-4 cells almost completely impaired collagen contraction.

In fibroblasts, contractility depends on RhoA-mediated signaling that results in myosin light chain (MLC) phosphorylation (Rhee and Grinnell, 2006; Totsukawa et al., 2000). Inhibiting the RhoA effector ROCK using the chemical reagent Y27632 likewise resulted in severely impaired collagen contraction in GFP control and GFP-syndecan-1 and -4 overexpressing MDA-MB-231 cells (III, Figure 3a). Although syndecan-4 was more efficient in contracting collagen matrix, on 3D collagen both syndecan-1 and -4 were found to co-localize with α2β1 integrin and actin in cell protrusions. In addition, MLC phosphorylation was higher in both GFP-syndecan-1 and -4 cells cultured in 3D fibrillar collagen compared to GFP control cells (III, Figure 3b, c). This difference was not seen on monomeric collagen (III, Figure 3b). α2β1 integrin and syndecan-1 and-4 are thus important for RhoA-mediated 3D collagen contraction in MDA-MB-231 breast cancer cells.

6. ECM invasion is dependent on the interplay between syndecans, integrins and MT1-MMP (III)

The oncogene K-Ras has been shown to influence the expression of many genes, including cell surface-bound *ITGA2* (α2-integrin), *SDC1* (syndecan-1), *SDC4* (syndecan-4) and the transmembrane collagenase *MMP14* (MT1-MMP) (Bild et al., 2006). All of these genes have been shown to be linked to invasion or adhesion to collagen and our aim was to study whether mutant K-Ras would control invasion via regulating the expression of these proteins in breast cancer. MDA-MB-231 cells are useful model cells because they carry a constitutively active mutation of K-Ras. RNAi-mediated silencing of *KRAS* indeed led to a downregulation of *ITGA2, MMP14, SDC1* and *SDC4* in MDA-MB-231 cells (III, Figure 1a) and invasion into both pepsin-

extracted and acid-extracted collagen was also impaired (III, Figure 1b, c). The method of isolating acid-extracted collagen preserves the naturally occurring crosslinks and cells are therefore dependent on the proteolytic activity of MT1-MMP when invading through this matrix. Pepsin-extracted collagen, on the other hand, lacks intermolecular crosslinks (Sabeh et al., 2004). We further examined the different functions of the K-Ras regulated proteins in invasion into these different collagen matrixes. Blocking α2-integrin significantly decreased invasion in both pepsin-and acid-extracted collagen (III, Figure 1d, 4a, b, 5a). Impairing MMP function either by using the pan-MMP inhibitor GM6001 or silencing MT1-2-and-3-MMPs also significantly decreased invasion in acid-extracted collagen compared to control Scr silenced cells (III, Figure 5a). This was measured by allowing treated cells to invade into cross-linked collagen for five days in the presence of siRNA or inhibitors. The gels were then processed into paraffin-embedded samples and stained with Hematoxylin and Eosin for analysis of invasion. Rather unexpectedly, syndecan-1 and -4 knockdown increased invasion by 2.7-fold and 3.3-fold, respectively (III, Figure 5a). Using this method of visualization we could also detect a difference in the mode of invasion: Syndecan-1-silenced cells invaded through the matrix as individual cells, while syndecan-4 silencing led to a more collective form of invasion in clusters. The differences in cell shape were also verified by analyzing morphological changes of syndecan-1 and -4 silenced cells embedded inside collagen plugs (III, Figure 5c). The distinct modes of cell invasion are possibly due to the observation that expression of syndecan-1, but not syndecan-4, inversely correlated with MT1-MMP expression (III, Figure 4c, d). Overexpression of GFP syndecan-1 resulted in 40% reduction of MT1-MMP levels (III, Figure 4c, d). Consistent with the knockdown experiments, GFP syndecan-1 and -4 expressing cells were able to invade significantly less into both pepsin- and acid-extracted collagen compared to GFP-cells (III, Figure 4a, b). Treating these cells with a ROCK inhibitor could further reduce invasion into pepsin-extracted collagen only in GFP syndecan-4 cells, but not in GFP syndecan-1 cells that already showed severely impaired invasion (approx. 61% reduction compared to GFP control) (III, Figure 4a). There is consequently a complicated interplay between integrins and syndecans in cancer cell invasion and remodeling.

DISCUSSION

1. Integrin traffic and cancer

Cell division failure and the formation of binucleate cells have been proposed to be triggering events in the formation of transformed cells. This is supported by the presence of tetraploidy in premalignant lesions (Galipeau et al., 1996; Olaharski et al., 2006). Correct integrin traffic is needed for execution of cytokinesis and our aim was therefore to study the consequences of derailed integrin traffic on tumor progression. Contrary to many studies on aneuploidy and cancer, we did not use drugs or chemicals to induce the cytokinesis failure, but employed a mutation that prevents normal cell division from taking place on specific matrices. Using several different assays we showed that cells with impaired integrin traffic become aneuploid and transformed *in vitro* and also form malignant tumors in mice. The observation that the euploid mutant β1YYFF_L0 mice were able to form aneuploid tumors also indicates that impairing integrin traffic can cause aneuploidy and transformation also *in vivo*. Although other integrins and ECM components should mediate normal cell division *in vivo*, the mutants may have difficulties undergoing cytokinesis, ultimately causing tumorigenesis. Tumor development would be seen after several cell generations, which would explain the long latency seen in the tumor growth of the β1YYFF_L0 cells. Although the β1YYFF mutation is not one found in cancers, these results nevertheless suggest a role for integrin traffic in tumorigenesis. The ovarian carcinoma cell line KFr13 which harbor a deletion in the *RAB21* locus form bi-and multinucleate cells in culture because of cytokinesis failure (Pellinen et al., 2008). Defects in integrin traffic may therefore be a causal factor in the tumorigenesis of these ovarian carcinomas and may be a reason behind genetic instability in other forms of human cancer as well. Furthermore, we also showed that prolonged silencing of Rab21 in HMECs resulted in binucleation and ultimately aneuploidy and that loss of Rab21 correlated with increased malignancy in prostate and ovarian carcinoma. In addition, several Rab GTPases that are important for intracellular trafficking are associated with cancer.

Induction of transient cytokinesis failure in p53 deficient cells leads to predisposition to transformation (Fujiwara et al., 2005; Ho et al., 2010). In addition to integrins, several proteins that participate in the cytokinesis machinery have been shown to be deregulated in cancer. Loss of heterozygosity of Plk4 is common in human hepatocellular carcinomas and $Plk4^{+/-}$ MEFs fail to execute cytokinesis and eventually become aneuploid and transformed (Rosario et al., 2010). Our results thus indicate roles for tumor suppressor genes and oncogenes in cytokinesis. In addition, budding yeast with a defective cytokinesis motor evolved pathways to restore growth by induction of aneuploidy and thereby increased gene expression of specific proteins needed for executing cytokinesis. Chromosomal variation thus likely drives the development of adaptive phenotypes (Rancati et al., 2008). Similarly, we also found that the aneuploid cells have regained the ability to traffic β1-integrin, which enables

cell division and probably contributes to their invasive abilities. A direct link between integrins and aneuploidy has not been established previously, although integrin function has been known to be important for cell division (Aszodi et al., 2003; Reverte et al., 2006). A fundamental question in this research area has been to elucidate the actual *in vivo* causes of aneuploidy and our results demonstrate that integrin traffic is involved in this process. This was demonstrated by our *in vitro* model and *in vivo* results. Deregulated integrin traffic has been shown to affect cancer cell invasion and impaired integrin traffic may thus enhance tumor progression by promoting both genomic instability and increased motility. FISH or flow cytometry that measures DNA content can be used to determine the presence of polyploidy, which should be used as a marker for failed cell division. The results of this research could also be taken advantage of clinically by studying integrin traffic, and the molecules that regulate this process, to detect premalignant stages of cancer. Especially concomitant decrease of p53 and Rab21 would indicate a propensity for aneuploidy. Integrin activation could be used to promote successful cell division by enhanced adhesion in cells that fail cytokinesis. Mouse models are often useful in cancer research, but transformation of mouse cells seems to involve fewer steps than transformation of human cells (Hahn et al., 1999). It is therefore important to take this research further and develop a reliable model to study how derailed integrin traffic impacts the transformation potential and gene expression of human cells with defective p53.

2. Consequences of derailed integrin traffic in MEFs

2.1 Chromosomal structural aberrations in the aneuploid MEFs

The aneuploidy that had arisen in the MEFs also seemed to involve some structural abnormalities. In tumorigenesis, there is more evidence for segmental aneuploidy, as opposed to only numerical changes in chromosomes (Fujiwara et al., 2005; Sotillo et al., 2007). However, in the CENP-$E^{+/-}$ model used by Weaver and co-workers there are no structural rearrangements, but aneuploidy nevertheless contributes to tumor initiation in this context (Weaver et al., 2007). One important question from our study is thus whether it is the aneuploidy as such providing the advantage or whether cytokinesis failure and subsequent genetic instability has a mutagenic effect leading to secondary mutations that provide a selective advantage. Structural errors in the aneuploid MEFs could for example lead to disruption of tumor-suppressor genes or generation of oncogenes. In these MEFs, DNA damage is relatively tolerated because of the lack of p53. It may also be that broken chromosomes are tolerated because of the presence of additional copies of the normal chromosome (Ganem et al., 2007; Duesberg et al., 2005). Thus, whether specifically numerical chromosomal deviations alone can lead to transformation is difficult to assess from these studies. Despite the structural aberrations detected by mFISH, there is nevertheless a lack of substantial genetic changes in the β1YYFF_L4 cell line according to the aCGH assay, and the genome seems to be structurally quite simple. The aCGH pointed to a few regions that were slightly gained or lost, but the major chromosomal differences between

β1YYFF_L4 and β1YYFF_L0 cells were overall numerical. This would support the conclusion that aneuploidy, i.e. gross genetic changes, and not specific focal gene deletions or amplifications, are driving the cancer. Exon sequencing could further be done to detect potential key driver mutations in these cells. In many tumor types copy number changes correlate with transcript abundance (Hyman et al., 2002; Pollack et al., 2002), but introduction of extra chromosomes has also been shown to lead to a destabilization of the whole transcriptome. For many of the highly overexpressed genes in β1YYFF_L4 no amplification was detected, suggesting that mainly gross chromosomal changes are causing the effect on gene expression.

2.2 Aneuploidy and chromosomal instability

The presence of aneuploidy was confirmed in the β1YYFF_L4 cells, but whether they are also chromosomally unstable is less clear. Aneuploidy and chromosomal instability (CIN) usually occur together in cells, but they are not synonymous. Aneuploidy refers to the state of a cell with an aberrant karyotype, while CIN refers to constant gains or losses of chromosomes at each cell division (Geigl et al., 2008). Some tumors do not have a CIN phenotype, but are stably aneuploid and contain cells with abnormal but similar chromosome sets. Whether these cells display another form of instability or whether they are in fact genetically stable is an important question when analyzing the role of numerical aneuploidies in cancer. The period of instability may also be transient until a stable clone with significant proliferative advantages outgrows the rest of the population (Storchova & Kuffer, 2008). A suggested outcome of excess centrosomes is increased rates of merotely and missegregation, which is thought to be the main cause behind the chromosomal instability seen in cancer cell lines with CIN (Thompson & Compton, 2008). Abrogation of p53 is additionally required for the maintenance of a stable CIN phenotype in an otherwise diploid population (Thompson & Compton, 2008**;** 2010**;** Yuen & Desai, 2008) and absence of p53 and Rb probably also allows for tolerance towards aneuploidy in the MEFs in these studies. Most β1YYFF_L4 cells undergo bipolar cell division, but the presence of merotely or lagging chromosomes has not been investigated. This would have to be confirmed by following the cells and chromosomes more closely through mitosis. Additional centrosomes can also lead to a multipolar mitosis and aneuploid daughter cells, although most cells that have undergone such massive changes in chromosome numbers are probably not viable (Nigg, 2002; Chandhok & Pellman, 2009). Occasionally, the β1YYFF_L4 cells also exhibit multipolar mitoses, in which three or four poles can be detected, but whether these cells actually survive has not been investigated. Nigg (2002) hypothesizes that tumor cell populations with multiple centrosomes expand through bipolar divisions, but maintain genetic instability by occasionally undergoing multipolar mitoses. The aneuploid β1YYFF cells appear to have a near-triploid, yet variable number of chromosomes, which would together with multipolar mitoses indicate the presence of CIN. This is suggested from chromosome counts and the observation that the 6 metaphases from the β1YYFF_L4 cells all had different chromosome numbers and there were variations in the copy number of individual chromosomes. However, as only 6 metaphases were made, it cannot be ruled out that there might be common

chromosome changes that would be detected with higher frequency. More metaphases from the β1YYFF_L4 and the control cell lines would have to be analyzed to draw conclusions about recurrent chromosome changes and the existence of chromosomal instability. In some epithelial cancer cell lines, there is evident structural and numerical chromosomal instability and cell-to-cell variability, but the composite karyotypes are relatively stable in the population during propagation in culture (Roschke et al, 2002). A composite karyotype depicts all clonal aberrations detected when combining FISH results from individual cells. Furthermore, it was recently shown in yeast that aneuploidy per se does not necessarily induce CIN, as different karyotypes displayed different degrees of instability (Zhu et al., 2012).

Thus, despite the indications of some level of instability, the altered karyotypes appeared quite stable in culture and two independent clones of laminin-treated β1YYFF cells showed triploid modal chromosome numbers, which might reflect the most favorable state of aneuploidy (Duesberg et al., 2005). In addition, the aneuploid cell lines β1YYFF_L4 and β1YYFF_L8 displayed a similar gene expression profile; all the aneuploid cells cluster together when studying general similarity between the samples. When comparing the β1YYFF_L4 and β1YYFF_L8 profiles very few genes are differentially expressed. These gene expression profiles and the constitutively high levels of specific proteins in the aneuploid populations appear to be very stable during long time culturing. The gained ability to endocytose integrins after four passages on laminin probably allowed for completion of cytokinesis and the propagation of a population of cells with an “optimal” expression profile. Without added selective pressure, perhaps the critical changes are conserved from one cell generation to the next, while dispensable chromosomes account for the variability seen between cells. It may be that the aneuploid β1YYFF cells have an optimal rate of CIN, i.e. they are plastic enough to display improved growth under suboptimal conditions and thus have an advantage over euploid cells. It is also important to bear in mind that the aneuploid cell line constitutes the whole population of cells that survived the laminin-treatment. Single-cell clones could be made to test for instability and expression profiles of the progeny of these clones.

2.3 Consequences of aneuploidy on cell fitness

There is somewhat of a paradox regarding aneuploidy in the sense that it is associated with cancer but also with reduced growth potential. Many studies on the consequences of aneuploidy have detected a stress response in the gene expression profiles and proteomic changes. Also, in most cases aneuploid cells show reduced fitness under normal growth conditions. This may result from abnormal levels of cell-cycle regulators (Stingele et al., 2012) or from a lack of energy, as much of the cells’ energy supply is supposedly needed to break down excessive proteins (Torres et al., 2007). For example, DNA or RNA metabolism has been shown to be downregulated. In the aneuploid β1YYFF MEFs, however, such a response was not detected in our gene expression profiling, although these cell lines showed decreased proliferative capacity on plastic compared to tetraploid MEFs. As aneuploid cells have been shown to be more reliant on mechanisms that relieve stress, sensitization could be done by targeting

the stress support pathways, such as the proteasome or autophagy. Another approach is to induce additional stress in these cells, by for example increasing genetic instability, so as to overwhelm the system. Both of these strategies would lead to either reduced growth or cell death (Luo et al., 2009; Tang et al., 2011). It would therefore be interesting to see if the aneuploid β1YYFF cells are more sensitive to Hsp90 inhibition and the stress inducing drug AICAR, which have been shown to be effective against trisomic MEFs (Tang et al., 2011).

The aneuploid β1YYFF MEFs displayed increased lipid metabolism, which has also been detected in tri-or tetrasomic human cells (Stingele et al., 2012). This increase in lipid metabolism could be due to the increased requirement for energy and the need to produce macromolecules from the precursors. The lack of other similarities in gene expression is probably due to the specific genetic context of the MEFs; induction of a stress response could be mild due to the lack of p53 and Rb. In addition, this stress response has been detected in yeast, mouse and human cells with an additional copy of one or more chromosomes. These models are very different to the massive aneuploidy that we see in the β1YYFF MEFs, which may account for rapid adaptation or such massive gene expression changes that this effect would not be detected. In a study on aneuploid yeast cells where no stress response was detected the cells also had multiple chromosomes in aneuploidy and had a heterogeneous karyotype (Pavelka et al., 2010; Sheltzer et al., 2011). Complex and simple aneuploidies may thus lead to very distinct responses. Furthermore, another clone of β1YYFF MEFs subjected to the same laminin-treatment also developed aneuploidy and tumorigenic properties. Although the aneuploidy-induced differentially expressed genes of this clone were associated with functions such as cancer, these cells did not display similar gene expression profiles as the β1YYFF_L4 cells that we have studied, indicating that both genetic context and random events dictate the changes induced by aneuploidy. In addition to increasing stress or targeting pathways needed to relieve stress, several strategies have been suggested when targeting aneuploidy. These also include exploiting factors that CIN cells are particularly dependent on as well as chromosome-specific targets such as potential driver genes or passenger genes that could be taken advantage of in therapeutics. In line with studies conducted in yeast, our results suggest that complex aneuploidies are in fact less stressed and may thus not respond to therapies that rely on this feature. Our study thus highlights the importance of analyzing the transcriptome and proteome and not only gene copy number changes, when determining the driving forces behind transformation and this should be taken into consideration when developing therapeutics.

3. Acquisition of malignant properties in aneuploid MEFs

Aneuploidy in itself is initially growth inhibiting and it may function as a selection barrier and thus promote improved proliferation in an aneuploid state (Torres et al., 2008). Derailed integrin traffic has enabled rapid evolution and selection to take place for genes and chromosome combinations that confer proliferative advantages and help

the cells adapt and survive. These cells have thus acquired many properties associated with cancer. By analyzing alterations in signaling pathways and gene expression we have found several changes that have evolved that contribute to this phenotype.

3.1 Evasion of apoptosis and self-sufficiency in proliferation

3.1.1 Role of the Src kinase pathway

As determined by serum deprivation or treating MEFs with TNF-α, the aneuploid cells are more resistant to cell death and are able to proliferate in the absence of important external stimuli. The increased levels of active Src may contribute to this phenotype as Src has a wide range of substrates and mediates many functions in cells. One target of Src is caspase-8, which is inactivated upon Src-mediated phosphorylation in HEK 293 cells. This suppresses Fas-induced apoptosis and Src may therefore contribute to tumorigenesis by downregulating the apoptotic machinery (Cursi et al., 2006). In addition, Src often cross-talks with RTKs and promotes signaling from them. In NIH-3T3 cell lines that overexpress EGFR, Src induces the expression of the anti-apoptotic protein Bcl-X_L and inhibiting Src can reverse the transformed phenotype of the cells (Karni et al., 1999). Src is also able to promote survival by, among other things, activation of the PI3K/Akt pathway or by activation of the mitogenic Ras MAPK cascade via FAK and the Grb2-Sos complex (Datta et al., 1996; Schlaepfer et al., 1996). However, there were no differences in Akt or Erk activity in detached cells nor in response to adhesion between the tetraploid β1YYFF_L0 and aneuploid β1YYFF_L4 cells, suggesting that Src and FAK would act via other pathways. To test the importance of the Src/FAK pathway in serum-free proliferation, inhibitors against these kinases could be added to this assay.

As cancer cells are able to proliferate in suspension, anchorage-independent growth is consequently used as an assay for detection of cell transformation. In epithelial cells, constitutively expressed FAK prevents anoikis and these cells are also able to grow in soft agar and form tumors in mice (Frisch et al., 1996). Src has also been implicated in protection from anoikis, as v-Src is able to suppress anoikis and induce anchorage-independency in human gallbladder epithelial cells and high Src expression correlates with resistance to anoikis in tumor cell lines (Hisano et al., 2003; Windham et al., 2002). Our results indicate that Src has a role in the anchorage-independency of the aneuploid cells, as Src inhibition significantly reduced proliferation in agarose after 72h. Interestingly, this effect could not be detected in adhered cells. This could be due to additional signals derived from ligated integrins and growth factors that are sufficient to promote proliferation and survival. The aneuploid cells may have developed a dependency on Src for proliferation in suspension, where integrin signals are lost. As determined by western blotting, the levels of active Src are higher in both adherent cells and detached aneuploid cells compared to euploid cells, which points to an uncoupling of Src from integrin signaling and constitutive activation of this kinase.

3.1.1.1 Regulation of Src

Our results show that upregulation of Eps8 and increased activity of EphA2 are at least partially responsible for the activation of Src. Eps8 consists of a PTB domain, proline rich sequences, an SH3 domain and an effector region in the C-terminus that interacts with actin (Gallo et al., 1997). There are two isoforms of this protein, a 97 kD and a 68 kD, which are thought to be generated by alternative splicing. Eps8 has various functions in the cell, including actin barbed end capping, actin bundling together with insulin receptor tyrosine kinase substrate 53 (IRSp53) downstream of Cdc42 and regulation of EGFR trafficking (Disanza et al., 2004; Disanza et al., 2006; Lanzetti et al., 2000; Scita et al., 2001). Complex formation of Eps8 and IRSp53 also leads to Akt, Erk and Stat3 activation (Liu et al., 2010) as well as Rac activation and increased cell migration (Funato et al., 2004). Increased expression has been detected in cervical and oral cancers (Chen et al., 2008; Chu et al., 2012) and v-Src kinase activity leads to Eps8 phosphorylation and induction of Eps8 expression (Gallo et al., 1997; Maa et al., 1999; Maa et al., 2007). Expression of Eps8, Src and FAK was found to correlate in a number of colorectal tumors (Maa et al., 2007). In colon cancer cell lines with high Eps8 expression, Eps8 silencing led to decreased levels of autophosphorylated active Src and decreased phosphorylation of the Src substrate Shc (Maa et al., 2007). Eps8 knockdown also decreased the levels of serum-induced pY397 and pY861 of FAK as well as total levels of FAK, and reduced proliferation and colony formation in soft agar. Eps8 was thought to induce FAK transcription via mTOR kinase and the transcription factor Stat3, which also regulates cyclin D1 levels. In addition, co-immunoprecipitation assays found FAK and Eps8 in the same complex and overexpression of FAK alone was sufficient to rescue the decreased proliferation and motility phenotype detected in Eps8-silenced cells (Maa et al., 2007).

In the absence of serum-derived stimuli, the activity of Src and FAK in the aneuploid MEFs seem to be dependent of the levels of Eps8, which are not affected by serum starvation. The SH3 domain of Src was shown to bind Eps8 *in vitro* and could therefore function as an activator of Src (Maa et al., 1999). The upregulated levels and peripheral localization of Eps8 could thus recruit Src to focal adhesions and lamellipodial structures where it together with FAK is able to phosphorylate several downstream targets. Knockdown of Eps8 in suspension and serum-free conditions also slightly reduced the levels of FAK pY397, but did not alter its localization to focal adhesions in adhered cells. This suggests that membrane recruitment of FAK is less Eps8-dependent in adhered cells, where other ligated adhesion receptors could promote FAK activation. Silencing of Eps8 in the aneuploid MEFs did not result in a clear phenotype on the actin cytoskeleton, but Eps8 could nevertheless take part in actin organization in these cells. As RhoA mediated stress fibers and actin polymerization has been shown to be needed for delivery of active Src to the plasma membrane (Timpson et al., 2001), Eps8 might participate in regulation of Src pY416 localization via actin.

Eps8 is able to bind to the NPXY motifs of β1 and β3 *in vitro* (Calderwood et al., 2003). Whether this binding is reduced due to the β1YYFF mutation has not been

established, although the Eps8 PTB domain binding has been suggested to be phospho-independent (Smith et al., 2006). One of the phenotypes of β1YYFF MEFs is reduced autophosphorylation of FAK Y397, which is necessary for complex formation with Src. This could be due to reduced interaction between mutant β1 and binding partners needed for FAK/Src activation, possibly Eps8. Eps8 and EphA2 were found at the same locations at the plasma membrane in the aneuploid MEFs. Being a transmembrane receptor, EphA2 could in association with Eps8 possibly enhance the recruitment of active Src to peripheral structures, because downregulation of both molecules had a similar effect on Src localization in the cell. The Eph receptors mediate bidirectional signaling via binding to membrane-bound Ephrin ligands on adjacent cells and this interaction usually promotes an epithelial phenotype. However, Eph receptors also mediate signals to oncogenic pathways and thereby promote cancer progression and this oncogenic function is often ligand independent. EphA1 overexpression was shown to promote transformation in mouse fibroblasts and EphA2 has been associated with malignancy in for example melanoma, glioma and breast cancer (Udayakumar et al., 2011; Zelinski et al., 2001). In glioma and prostate cancer, ligand-independent Akt-mediated phosphorylation of EphA2 induces migration and invasion while EphA2 ligation with Ephrin-A1 inhibited cell motility (Miao et al., 2009). In lung squamous cell carcinoma an activating mutation of EphA2 caused increased phosphorylation of Src, cortactin, $p130^{Cas}$ and mTor, as well as increased apoptosis-resistance, anchorage-independent growth and invasion (Faoro et al., 2010). Thus, the reduced ability to activate the Src/FAK complex in β1YYFF MEFs may be what actually drives the cells towards overcoming this barrier. This seems to have been achieved at least partly via Eps8 and EphA2 resulting in activation of and selection for this signaling pathway. These data broaden our understanding of the regulatory mechanisms that control Src activity and suggest a role for Eps8 and Src co-operation in cancers other than colorectal.

3.1.2 Role of the transcription factor Twist2

In this study, we also found increased expression of Twist2 in the aneuploid MEFs, as well as increased nuclear localization. Expression of Twist2 was also confirmed in a subset of clinical tissue samples from cancer patients. In addition, we detected Twist2 in sarcomas, suggesting that Twist2 proteins could be important for the tumor progression of not only carcinomas, but also cancers of mesenchymal origin. Interestingly, Twist1 was also found to be expressed in 8/15 cases of analyzed rhabdomyosarcomas (Maestro et al., 1999). In these tissue samples, surrounding normal cells were Twist1 negative. The Twist transcription factors belong to the basic Helix-Loop-Helix (bHLH) transcription factor family. Twist2 is closely related to Twist1 as they are more than 90% similar in the C-terminal region where the basic, HLH and Twist box domains are (Franco et al., 2011; Li et al., 1995). There are more differences in the N-terminus, which probably allows for different interaction partners. The N-terminal regions also consist of nuclear localization signals. The two transcription factors have separate and redundant functions and they are able to form both homo- and heterodimers. The Twist proteins recognize E-box responsive elements via the bHLH region and act as either activators or repressors of transcription. They are

important for many developmental processes and transcriptional activation of especially Twist1 is mediated by various signaling molecules and other transcription factors, including TGF-β, Wnt, Insulin receptor and N-myc (Dong et al., 2007; Valsesia-Wittmann et al., 2004). In addition, they have been proposed to have a general role in tumor progression which is related to their protective role against apoptosis and induction of senescence, or promoting proliferation. Twist2 has been detected in breast cancer, melanoma and several SCCs, and its expression correlates with poor survival in some cancer types (Ansieau et al., 2008; Fang et al., 2011; Gasparotto et al., 2011; Li et al., 2012; Liang et al., 2011; Mao et al., 2012; Zhou et al., 2012).

3.1.2.1 Twist proteins in apoptosis and senescence

Cancer cells often protect themselves against oncogene-induced apoptosis by additional genetic alterations that render them resistant to this induction. In Rat-1 cells and MEFs expression of both Twist1 and Twist2 protected cells from apoptosis induced by the Myc or Ras oncogenes, or serum withdrawal (Maestro 1999). The increased survival was thought to be due to Twist-mediated downregulation of the p53 activator ARF (Maestro et al., 1999; Valsesia-Wittmann et al., 2004), but Twist has also been shown to interfere with the p53 pathway by for example direct binding between the two transcription factors (Piccinin et al., 2012; Shiota et al., 2008). Both aneuploid and tetraploid MEFs lack p53, however, and the mechanism of increased apoptosis resistance is probably p53-independent. Twist1 and -2 are also induced by NF-κB and regulate the NF-κB survival pathway in a negative feedback loop. They inhibit NF-κB-dependent induction of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Twist2$^{-/-}$ cells consequently display an upregulation of TNF-α and are also more sensitive than wild-type cells to dose-dependent TNF-α-induced apoptosis (Sosic et al., 2003). Twist1 and 2 have also been shown to protect from apoptosis induced by the DNA replication inhibitor drug daunorubicin by preventing inhibitory phosphorylation of the anti-apoptotic protein Bcl-2 (Pham et al., 2007). This protection was p53 and ARF-independent. In addition, Twist1 knockdown in A549 lung carcinoma cells also sensitized these cells to cisplatin-induced apoptosis presumably via decreasing levels of Bcl-2 and increasing levels of pro-apoptotic Bax and pJNK. In addition to Dusp4, Twist2 could thus have a role in the cisplatin resistance detected in the aneuploid MEFs. However, we did not detect any increased sensitivity in TNF-α induced apoptosis in Twist2-silenced aneuploid cells compared to control silenced cells, suggesting that the level of knockdown was not enough to sensitize the cells, or that other mechanisms have evolved to protect these cells from cell death. Apoptosis-resistance could also partly be due to the downregulation of the TNF receptor superfamily member 6 (Fas) (-3.3) or caspase 7 (-3.4) detected in β1YYFF_L4. Fas is a well-known death receptor that is activated by binding to FasL and it is important in mediating, among other things, T-cell cytotoxicity (Schulze-Osthoff et al., 1998). Death signals mediated via both Fas and TNF-R1, the receptor for TNF-α, activate the caspase cascade and the increased tolerance towards apoptosis could be due to deregulation of the apoptotic machinery. The mechanisms mediating this resistance may on the other hand not necessarily be seen on the gene expression level.

Twist2 was found to be upregulated in a large fraction of tumors developing in the MMTV-*ErbB2/Neu* transgenic mouse model. Interestingly, knockdown of Twist2 in cell lines derived from these tumors led to growth arrest and oncogene-induced senescence (Ansieau 2008). Both Twist1 and -2 knockdown also led to induction of senescence in human breast cancer and melanoma cell lines. Especially melanoma was found to have high expression of both proteins. Also overexpression of H-RasV12 and Twist1 or Twist2 in primary MEFs resulted in formation of tumors in mice and growth in soft agar, whereas expressing H-RasV12 alone led to senescence. Ras induces expression of $p16^{Ink4a}$ and $p21^{Cip1}$, but the Twist proteins inhibited this promoter activation. Although the β1YYFF_L0 and β1wt MEFs used in this study are SV40 Large T immortalized, they were not able to form large colonies in soft agar after introduction of constitutively active H-RasV12. However, the aneuploid MEFs were more susceptible to Ras-induced transformation, suggesting cooperation between Ras and other molecules induced by the aneuploidy, potentially Twist2 or Src, to promote the formation of large colonies in soft agar and increased foci formation.

Furthermore, Twist1 and Twist2 are associated with self-renewal capabilities characteristic for stem cells and overexpression of these proteins in epithelial cells increases the ratio of $CD44^{high}/CD24^{low}$ cells in the population (Fang et al., 2011; Isenmann et al., 2009; Mani et al., 2008). Interestingly, the stem cell markers CD44 was also slightly upregulated on the gene expression level in the aneuploid MEFs, linking aneuploidy to the induction of stem cell-like properties.

3.1.2.2 Twist2 and the lipid metabolic pathway

The aneuploid MEFs displayed increased expression of many genes involved in lipid metabolism and specifically in biosynthesis of cholesterol. Twist2 has also been linked to metabolic functions, as Twist2 knockout mice display adipose deficiency and reduced glycogen storage and energy metabolism (Sosic et al., 2003). Also, Twist2 was shown *in vitro* to bind to and inhibit the transcriptional activity of Srebp1a and c (generated by alternative splicing of the Srebp1 gene) via histone deacetylase activity (Lee et al., 2003). However, in this study there was no effect of Twist2 on the transcriptional activity of Srebp2 (Lee et al., 2003). Interestingly, we found that Twist2 silencing in the aneuploid MEFs led to decreased expression of many Srebp1/2 controlled genes, including Dhcr24, Cyp51, Fdps, Mvd, Hmgcs1, Nsdhl and Srebf2. Many of the genes affected are part of the cholesterol biosynthetic/mevalonate (MVA) pathway which in several recent studies have been associated with transformation (Clendening et al., 2010; Freed-Pastor et al., 2012; Ros et al., 2012). Expression of Hmgcr in various cell lines increased the cells' ability to grow in soft agar but had no effect on cell death. High mRNA levels of Hmgcr, Hmgcs1, Mvd, Acat2, Nsdhl and Fdps also correlated with poor survival of breast cancer patients (Clendening et al., 2010; Freed-Pastor et al., 2012). The MVA pathway has also been shown to be controlled by mutant p53 and responsible for the characteristic 3D morphology of breast cancer cells. MVA pathway inhibition by statins consequently induced a reversal of this phenotype to a normal-like acinar shape. These effects in 3D Matrigel cultures

are thought to be due to decreased growth and invasion as well as increased cell death (Freed-Pastor et al., 2012).

The metabolic switch often detected in tumor cells facilitates production of biomass and supports the anabolic requirements of cell growth. The mevalonate pathway is required for synthesis of among other things cholesterol, isoprenoids and isopentenyladenine and it is thought that tumor cells are more dependent on the metabolites generated by this pathway. Membrane cholesterol and a specific ceramide composition in the membrane are required for cell division. Inhibition or downregulation of glucosyl ceramide synthase, which functions in one of the last steps of sphingolipid synthesis, leads to failed cytokinesis because of mislocalization of actin and ERM (ezrin radixin moesin) proteins (Atilla-Gokcumen et al., 2011). Also, farnesylpyrophosphate and geranylgeranyl pyrophosphate are posttranslationally added to small GTPases and are essential for proper signaling. The link between Twist2 and the mevalonate pathway is therefore intriguing and its role in the proliferation of the aneuploid cells is emphasized by the observation that the Srebp inhibitor Fatostatin had a similar effect on anchorage-independent growth as knockdown of Twist2. Furthermore, the aneuploid cells also displayed upregulation of Scd1, Scd2 and Elovl6 genes, which are part of the de novo fatty acid synthesis pathway which is often deregulated in cancer (Freed-Pastor et al., 2012; Hilvo et al., 2011; Menendez and Lupu, 2007). The metabolic alterations associated with aneuploidy are thus likely to play a role in the malignancy of the cells. Transcription factors are difficult drug targets due to their broad range of functions and nuclear localization. Targeting the lipid metabolic pathways using available drugs could thus be done in Twist2 expressing cancer cells. The relationship between the lipid biosynthetic pathway and Twist2 should nevertheless be further studied in order to pinpoint the exact nature of this relationship and its role in tumor progression.

3.2 Invasion

The dramatically increased invasive ability gained by the aneuploid cells is at least partly due to the specific gene expression and signaling changes detected in these cells. Src and FAK are involved in normal regulation of cell-matrix adhesions and cell migration by influencing among other things Rho GTPases. v-Src transformed cells usually have weakened cell-ECM contacts, exhibit increased motility due to altered focal adhesion turnover and increased invasion *in vivo* (Fincham and Frame, 1998; Playford and Schaller, 2004). Like Src, FAK is rarely mutated in cancer, but high expression is linked to malignancy and may have an important role in tumor invasion and metastasis (Mitra and Schlaepfer, 2006). Inhibiting FAK in NIH-3T3 fibroblasts disrupts the v-Src/FAK complex and suppresses v-Src induced invasion in Matrigel and metastasis *in vivo*, but has no effect on the ability of v-Src to induce anchorage-independency or tumor formation in mice (Hauck et al., 2002). Also, v-Src expressing fibroblasts lacking FAK altogether are motile, but not invasive (Hsia et al., 2003). The effect of inhibition of FAK activity on specifically the invasiveness of the aneuploid cells would therefore be of interest. The Src/FAK complex is thought to promote invasion by activating Rac and JNK and thereby induce upregulation of matrix

metalloproteinase 9 (MMP-9) and MMP-2, which degrade the ECM and facilitate invasion (Hsia et al., 2003). Src inhibition may therefore reduce the invasive capacity of the aneuploid cells by e.g. influencing Src/FAK-mediated regulation of the actin cytoskeleton or induction of MMPs. Interestingly, the aneuploid cells also seemed to display increased activation of RhoA. However, treating the β1YYFF_L4 cells with RhoA, Rho kinase or Rac inhibitors had no dramatic effect on invasion, which could be due to inefficient inhibition or the dependency on other pathways.

In addition to enhancing proliferative capacity, the Twist transcription factors have been shown to promote motility and invasiveness in cancer cells and especially the well-studied Twist1 protein is highly associated with EMT and metastasis (Ansieau et al., 2008; Fang et al., 2011; Li et al., 2012; Mironchik et al., 2005; Soini et al., 2011; Yang et al., 2004). The Twist proteins are therefore suggested to favor transformation by concomitantly overriding failsafe programs and inducing EMT. However, in a mouse mammary tumor model, knockdown of Twist1 specifically inhibited lung metastasis, but had no effect on proliferation of the primary tumors (Yang et al., 2004). Twist1 is able to promote metastasis in breast cancer cells by inducing expression of miR-10b, which results in downregulation of HOXD10 and concomitant upregulation the invasion-promoting small GTPase RhoC (Ma et al., 2007). Interestingly, in MDA-MB-231 cells stimulation with hyaluronan (HA) induced a complex formation of CD44 and phosphorylated Src. This subsequently led to Twist1 phosphorylation and translocation to the nucleus, where induction of miR-10b could take place. In addition to increased expression of RhoC, HA treatment also ultimately resulted in elevated levels of RhoA and increased invasion in Matrigel-coated transwell units (Bourguignon et al., 2010). Twist1 and miR-10b have also been shown to upregulate expression of MT1-MMP via HOXD10 in breast cancer tumor initiating cells. MT1-MMP translocation to the cell surface was induced in these cells in response to hypoxia and this enhanced invasion both *in vitro* and *in vivo* (Li et al., 2012). Similarly to Twist2, microRNA-10b was also upregulated in the aneuploid MEFs compared to tetraploid control. Interestingly, downregulation of Twist2 in the aneuploid MEFs led to decreased expression of miR-10b, demonstrating that this microRNA is controlled by both Twist transcription factors. However, anti-miR-mediated inhibition of miR-10b in the aneuploid MEFs was not sufficient to reduce invasion in Matrigel. This could be due to the low efficiency (~50%) of inhibition, or due to other signaling routes employed by Twist2 to drive invasion. Furthermore, blocking CD44 or silencing Has1 in these cells had no apparent effect on invasion, indicating that the aneuploid cells are not dependent on these molecules for their motility.

The metabolic changes detected in the aneuploid cells may also affect their invasive ability and the effect of Twist2 on the cholesterol synthesis pathway is another plausible candidate for increased invasion. Inhibition of especially geranylgeranyl transferase had a large effect on both growth and invasion of MDA-MB-231 cells in 3D Matrigel. In MDA-MB-231 cells with a deficient MVA pathway due to inhibition of mutant p53, addition of geranylgeranyl-pyrophosphate restored their invasive ability (Freed-Pastor et al., 2012). It would be interesting to see how Fatostatin affects

invasion of the aneuploid MEFs and how addition of different metabolites affect invasion in Fatostatin-inhibited cells. In addition, the MEFs are dependent on β1-integrin for invasion as demonstrated by function-blocking antibodies. The aneuploid MEFs have somehow regained the ability endocytose beta integrin, most likely via a clathrin-independent route. Cholesterol is essential for caveolae-dependent endocytosis and increased cholesterol production in the MEFs could therefore potentially enhance integrin internalization via this pathway. The lipid metabolic pathway may thus prove to be an essential target in cancer therapy due to its multifunctional role in cancer.

4. Syndecans in cancer cell invasion

In this study we found that the oncogene K-Ras regulates the expression of integrin α2β1, syndecan-1 and -4 and MT1-MMP in MDA-MB-231 breast carcinoma cells. Syndecan-1 and integrins have been shown to cooperate during cell adhesion to 2D surfaces in different cell models (Ishikawa and Kramer, 2010; Vuoriluoto et al., 2008). Our results indicate that in 3D environments the interaction between these adhesion receptors may be different. For example, we found that both syndecan-1 and syndecan-4 co-localize with integrin and actin on 3D collagen, while co-localization was detected only for syndecan-1 on 2D (Vuoriluoto et al., 2008). This study also shows that α2β1 integrin, syndecan-1 and syndecan-4 are important for RhoA-mediated 3D collagen contraction. Interestingly, these syndecans were found to be negative regulators of invasion as knockdown of syndecan-1 and -4 increased invasion in collagen, although by induction of different morphological changes.

4.1 Syndecan expression in cancer

Syndecan knock-out mice exhibit only mild phenotypes, indicating that the different syndecans probably have redundant functions during development. However, syndecan-1 and -4 null mice show defects in for example wound healing (Echtermeyer et al., 2001; Stepp et al., 2002). In addition, syndecan expression has been shown to have a role in different diseases, including HIV pathogenesis, obesity, myocardial infarction and cancer (Bobardt et al., 2003; Fears and Woods, 2006; Finsen et al., 2004). The role of syndecan in cancer is not an unambiguous one, especially regarding syndecan-1 which is the most studied member of the syndecan family. Increased expression has been detected in some human cancers, including glioma, pancreatic, prostate, and breast cancer and has been linked to poor prognosis (Barbareschi et al., 2003; Barbareschi et al., 2003; Chen et al., 2004; Conejo et al., 2000; Lendorf et al., 2011; Zellweger et al., 2003). However, in clinical samples of head and neck squamous cell carcinoma high syndecan-1 correlated with smaller primary tumors and a more differentiated phenotype (Inki et al., 1994). In ovarian, colorectal, skin and lung cancer, syndecan-1 is likewise decreased (Fujiya et al., 2001; Shinyo et al., 2005; Stepp et al., 2010). Furthermore, there is discrepancy among different syndecan-1 studies on the same cancer type, which could reflect factors that affect syndecan expression such as heterogeneity of the surrounding matrix or the absence or presence of angiogenesis at

the tumor site. Syndecan-2 is mostly upregulated in neoplastic tissues, including colon and ovarian cancer (Davies et al., 2004), while syndecan-4 is involved in adhesion and spreading of breast cancer (Fears and Woods, 2006). In one study with breast carcinoma samples, syndecan-1 and syndecan-4 seem to have opposing or at least independent roles. Syndecan-1 expression correlated with increased malignancy and poor prognosis, while syndecan-4 was present in estrogen and progesterone receptor - positive tumor samples (Lendorf et al., 2011). Also, these syndecans seemed to be mutually exclusive in the samples that showed correlation between syndecan expression and tumor grade or hormone receptor status.

4.2 Syndecan-mediated inhibition of cell invasion

The inhibition of cell invasion that we detected in syndecan expressing MDA-MB-231 cells could occur via different mechanisms. The GAG chains of syndecans bind many growth factors and thereby facilitate ligand binding to the corresponding receptor. Although depletion of GAG chains has been shown to cause reduced growth factor signaling, syndecan knockdown would also result in the release of signaling molecules into the matrix which could enable invasion-promoting signaling. As syndecans participate in integrin-mediated interactions with the extracellular matrix, syndecans might enhance the adhesive properties of cells leading to retarded migration and invasion. Integrin traffic is important for cancer cell invasion and syndecan-4 has been shown to regulate α5β1 integrin endocytosis, which is needed for cell migration and wound closure (Bass et al., 2011). Also, α5β1 integrin recycling and cell invasion is increased upon inhibition of αvβ3 integrin (Caswell et al., 2008) and syndecan-1 has been shown to activate αvβ3 (Beauvais et al., 2004; Beauvais and Rapraeger, 2010). Syndecan-mediated regulation of integrin traffic could possibly lead to a tilt in the balance of optimal internalization and recycling rates needed for motility, leading to reduced invasion in syndecan expressing breast cancer cells. Syndecans contain many domains that are important for interaction with binding partners, especially the actin cytoskeleton that is essential for cell motility. Both Rac1 activity at the front of the cell and RhoA-mediated contractility at the rear are necessary for successful invasion. Because syndecans participate in the fine-tuning of Rho GTPase signaling, disruption of this regulation could decrease invasion in 3D. For example in head and neck squamous cell carcinoma syndecan-1 silencing reduced RhoA activation, but increased invasion in collagen I (Ishikawa and Kramer, 2010).

The different modes of invasion detected in syndecan-1 and syndecan-4 silenced cells nevertheless indicate that these syndecans increase invasion via distinct mechanisms. Syndecans are also able to shed their ectodomain, by for example MT1-MMP that cleaves off the extracellular part, and this soluble domain is often detected in the serum of cancer patients. Shed ectodomains can bind to growth factors or cytokines and thus promote tumorigenesis by inducing cell migration towards these factors. They can also activate angiogenic factors, protect proteases from inhibition and compete for ligands with surface-bound syndecans, thereby modulating signaling. In contrast to soluble syndecan-1, membrane-bound syndecan-1 was shown to inhibit invasion in Matrigel in MCF7 breast cancer cells (Nikolova et al., 2009), which is consistent with our results.

We also showed that syndecan-1 expression reduced the levels of MT1-MMP, which leads to speculation whether syndecan-1 regulates its own cleavage by suppression of MT1-MMP expression. The increase in MT1-MMP in syndecan-1 silenced cells can facilitate individual cell invasion by direct degradation of the matrix which enables forward cell movement, by activating other adhesion receptors or proteases, by releasing serum-derived growth factors or by making matrix epitopes available which promote this mode of invasion. Interestingly, E-cadherin expression in mammary tumors correlated with syndecan-1 expression (Leppa et al., 1996), which is line with our results on induction of individual migration in syndecan-1 silenced cells. Also, miR-10b dependent downregulation of syndecan-1 in breast cancer cells led to increased invasion in Matrigel and syndecan-1 depletion reduced the levels of E-cadherin (Ibrahim et al., 2012).

The syndecan-4 silenced cells, on the other hand, seem to invade as a cohort and have to some extent maintained cell-cell-contacts. These contacts enable force transmission between the cells and could be maintained by tight-junction proteins, gap junction proteins, cadherins and integrins. Syndecan-4 silenced cells are probably less dependent on proteolysis, with exception for the leading cell of an invading cell cohort. Also, pericellular proteolysis has been shown to be needed for widening the invasive track of collectively invading cells (Wolf et al., 2007). The nature of the cell-cell junctions could be examined by additional immunostaining and 3D microscopy. Time-lapse imaging of invasive cells in both *in vitro* and *in vivo* systems would also be a useful tool to study the dynamics of the cell morphology in syndecan silenced cells. In conclusion, our studies indicate that syndecans mediate protective functions against metastasis of breast cancer cells and syndecans could be used as a tool for preventing metastatic spread. In contrast to studies on 2D collagen, Syndecan-4 is a co-receptor of $\alpha 2\beta 1$ integrin in 3D collagen, which demonstrates that the collaboration between integrins and syndecans depend on the dimension and structure of the matrix.

SUMMARY AND CONCLUSIONS

In this thesis we set out to determine the role of integrin traffic in both tumorigenesis and cancer cell invasion. Our studies have shown that mere inhibition of integrin traffic in MEFs is sufficient to cause oncogenic transformation; failed integrin traffic has induced a switch in these cells from tetraploid to aneuploid, from non-invasive to invasive, from growth factor- and substrate –dependent to anchorage-independent and resistant to apoptosis and serum deprivation. These results thus indicate a causative role for aneuploidy in tumorigenesis and specifically that complex aneuploidies, which are the result of cell division failure, in fact are more tumorigenic under certain conditions than aneuploidy arising from diploid cells. A key question in drug design is whether all aneuploid cancers develop their own strategy to adapt to the abnormal genome and enable proliferation or whether aneuploid cells share common changes. Our studies show that both genetic context and stochastic events determine the course of tumor development. By triggering the development of alternative pathways, aneuploidy provides a means to overcome mechanisms that confer a proliferative disadvantage to cells. The complexities of genetic changes that take place during tumor evolution emphasize the need for personalized medicine in cancer treatment. A more comprehensive understanding of the consequences of aneuploidy is still required for developing new therapeutics.

By studying the function of the cell surface receptors integrins and syndecans in 3D collagen we found that syndecan-1 and -4 are important for matrix contraction and syndecan-4 co-operates with α2β1 integrin in this process. The oncogene K-Ras contributes to invasion of breast cancer cells and also regulates the expression of the genes encoding for α2 integrin, MT1-MMP and syndecan-1 and -4. Both syndecan-1 and -4 were found to be negative regulators of breast cancer cell invasion. These results give new insights into the relationship between molecules involved in the invasive process and are an important step in characterizing pathways regulating the events that enable metastasis. The differences in cell behavior detected between 2D and 3D assays also highlight the importance of engineering more tissue-like matrix structures to recapitulate the *in vivo* environment in *in vitro* models.

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